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Comparative Lipid and Lipoprotein Metabolism

John E. Bauer¹

This review summarizes a number of recent reports in several areas of lipid and lipoprotein metabolism. Absorption of dietary lipids, cholesterol synthesis, and biliary cholesterol metabolism are mentioned only briefly to be complete. Comparative aspects of lipoprotein metabolism, however, are detailed in an effort to integrate the myriad metabolic events which characterize these important lipid transport particles. Where comparative information is known, those aspects of lipoprotein metabolism that may be protective against atherogenesis in certain mammalian species are also described. Efforts to understand atherogenic resistance comparatively in animals lends a better understanding of the metabolic events leading to coronary artery disease in humans. They also provide an important basis for understanding lipid metabolism in numerous veterinary species.

Key Words: Lipid, lipoprotein, cholesterol, comparative, atherogenesis

Absorption of Dietary Lipids

It is estimated that humans ingest about 1 g of cholesterol daily with an equal amount available endogenously from intestinal lumen secretions (bile) and sloughed cells. Uptake of free cholesterol occurs from mixed micelles, primarily in the jejunum, most of which is esterified with a fatty acid.¹ Before absorption, the esters are hydrolyzed by pancreatic cholesterol esterase within the lumen. Intestinal mucosal cell resynthesis of cholesteryl esters then occurs mediated by acyl-CoA:cholesterol acyltransferase (ACAT). These esters plus free cholesterol are secreted as components of chylomicron particles. High cholesterol diets result in increased ACAT enzyme activities suggesting a relationship between cholesterol absorption and this enzyme.

In the post-prandial state, triglycerides (TG) are also hydrolyzed in the small intestinal lumen via pancreatic lipase which releases fatty acids, mono- and diglycerides. Combined with bile acids, cholesterol, and phospholipids, these components form mixed micelles that transfer the lipids to adjacent mucosal cells for absorption. Within the cell, re-esterification of TG occurs. This TG, along with cholesteryl esters, will form the core of the subsequently synthesized chylomicron particle, with free cholesterol, phospholipids, and one of the apo-B proteins, apo-B₄₈ as surface components.

De Novo Cholesterol Biosynthesis and Biliary Cholesterol Metabolism

Virtually all nucleated cells can synthesize cholesterol whereby acetyl-CoA is converted to 3-hydroxy-3-methylglutaryl Co-enzyme A (HMG-CoA). This compound is reduced to mevalonic acid by virtue of the enzyme HMG-CoA reductase, the rate-limiting step of cholesterol biosynthesis. There has been considerable interest in this enzyme since a number of pharmacologic compounds have been found to modify its activity. Drugs that inhibit the activity of HMG-CoA reductase in combination with the bile acid binding resin, cholestyramine, can be particularly effective in helping reduce serum cholesterol concentrations in hypercholesterolemic patients. Thus, not only is cholesterol biosynthesis inhibited, cholestyramine ingestion results in bile acid binding and increased fecal excretion. The bile acids are thus unavailable for resorption which shifts the cholesterol pool in favor of increased hepatic bile acid synthesis. Our studies using rabbits have shown that animals hyperresponsive to dietary cholesterol have increased biliary free and esterified cholesterol concentrations.³ This finding is noteworthy because enterohepatic recirculation of biliary contents may exacerbate the intestinal load of endogenous and exogenous cholesterol presented for absorption. Under certain conditions, this recircula-

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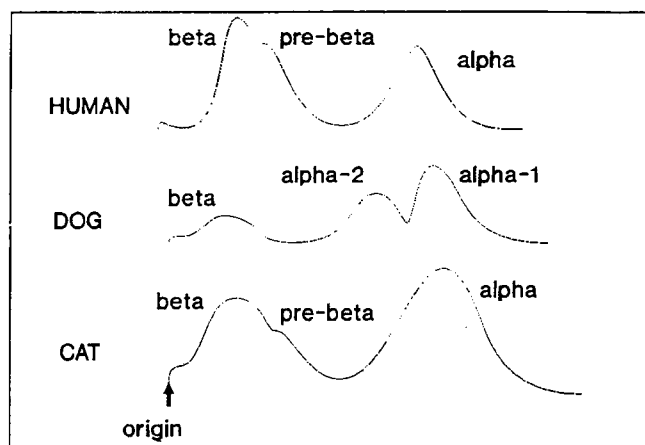


FIG. 1 — Densitometric tracings of lipoprotein electrophoretograms: Human, dog, and cat patterns stained with Fat red 7B after electrophoresis on 1% agarose gels are as shown. The horse pattern is similar to that of cats, the rodent pattern is often similar to that of dogs; all of these species are HDL mammals. The pattern for LDL mammals including rabbits and monkeys, etc., after high dietary fat/cholesterol feeding is similar to that seen in humans. Figure used with permission, from Bauer JE, JAVMA 201(11):1691-1694, 1992.

tion of cholesterol may contribute to the hypercholesterolemic state.

Cholesterol synthesis can also be down regulated via low density lipoprotein receptor mediated delivery of lipoprotein cholesterol. This phenomenon is partially controlled by the expansion or contraction of hepatic cholesterol pools resulting in a coordinate balance among hepatic cholesterol biosynthesis, biliary secretion of cholesterol and bile acids, and receptor uptake.

Comparative Lipoprotein Metabolism: Structure and Function

LIPOPROTEIN CLASSES

Five major types of lipoproteins (LP) are known. These include chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). The less dense particles tend to be larger, and contain more lipid and less protein. Heterogeneity within each class is recognized and designations using subscripts such as HDL₂ and HDL₃ have been used to refer to such subclasses. Chylomicrons are the largest LP particles. They function to transport intestinally absorbed lipids in the circulation. The other classes (VLDL, IDL, LDL, HDL) are involved in endogenous lipid transport either to or from tissues and are metabolically subjected to receptor and non-receptor mediated metabolic processes, enzymatic, and lipid exchange reactions.

The lipoproteins are, for the most part, viewed as spherical micellar particles; hydrophobic triglyceride and cholesteryl esters comprise a core surrounded by an

TABLE 1
Low Density Lipoprotein Characteristics
of Selected Species

Lipoprotein	Species	Hydrated Density (g/ml)	Electrophor Mobility	Major Apo-Proteins
Chylomicron	Human	0.930	origin	B ₄₈ , A, C, E
	Dog	"	"	"
	Cat	"	"	"
	Rodent	"	"	"
	Rabbit	"	"	"
	Monkey	"	"	"
VLDL	Human	<1.006	pre-β	B ₁₀₀
	Dog	"	"	B ₁₀₀ , B ₄₈ *
	Cat	"	"	B ₁₀₀
	Horse	"	"	B ₁₀₀ , B ₄₈ *
	Rodent	"	"	B ₁₀₀ , B ₄₈ *
	Rabbit	"	"	B ₁₀₀
LDL	Human	1.019-1.063	β	B ₁₀₀
	Dog	1.019-1.087	"	"
	Cat	1.019-1.087	"	"
	Horse	1.016-1.063	"	"
	Rodent/Rabbit	1.019-1.063	"	"
	Monkey	1.019-1.063	"	"

*Hepatic apolipoprotein B₁₀₀ mRNA editing has been demonstrated in this species.¹⁸

amphipathic coat of phospholipids, unesterified cholesterol and proteins. These structures are in a dynamic state of equilibrium with respect to each other and the various tissue and cellular compartments. As such, transfer and exchange reactions of individual components can occur, but not to the same extent or rates. Generally, the more water insoluble components require specific transfer factors to facilitate their movement between particles. Cholesteryl ester transfer protein is an example of one such factor. The proteins (apoproteins) tend to be specific and unique to a particular LP class although some are in common due to exchange or precursor-product relationships depending upon metabolic phenomena in which they participate. In spite of these interrelationships, certain functions are recognized for each class of LP. Thus, they differ in size, composition, hydrated density, apolipoprotein content, site of formation, and electrophoretic mobility (Tables 1 and 2, and Fig. 1). Nomenclature and classification based on these characteristics are most widely recognized and developed in the scientific literature. An extensive review of comparative mammalian lipoprotein analyses has been published.⁴

LDL VS. HDL MAMMALS

Although no species is identical to humans regarding every element of lipid and lipoprotein metabolism, some species have similar characteristics. These serve as models to address specific questions. For example,

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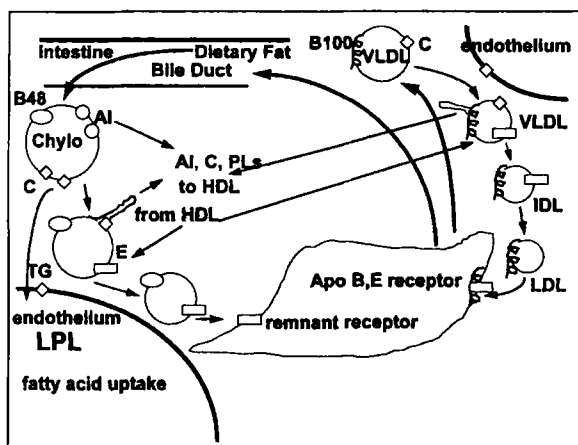


FIG. 2 — Chylomicron, VLDL and LDL metabolism: Dietary fat, digested in the small intestine, is incorporated into chylomicrons containing apo-B₄₈ which travel via the lymphatics system to the circulation. Here the particles acquire apolipoproteins (apo-E and apo-C) from high density lipoproteins and lose their triglyceride via hydrolysis by lipoprotein lipase (LPL) in adipose and striated muscle capillary endothelia. Fatty acid uptake thus occurs. Remnant particles formed in this fashion are removed by a cell-surface receptor that binds apo-E. VLDL are assembled in the liver and secreted into the circulation and also acquire apo-E and apo-C peptides. These lipoproteins are similarly metabolized via LPL leaving triglyceride-depleted remnant particles such as IDL which will ultimately form triglyceride-poor, cholesterol-rich LDL. LDL and their associated cholesterol can be taken up by the liver and other cells via apo-B, E receptor mediated events.

certain mammalian species have a predominance of LDL, much like humans, especially under certain dietary conditions. These include rabbit, hamster, guinea pig, pig, camel, rhinoceros, and most monkeys. As a whole, this group is sensitive to diet-induced elevations in LDL cholesterol. Although the predominant LP in some of these animals in their natural environment is HDL, their LDL fractions readily respond to high dietary fat and cholesterol consumption. Because of the association between diet-induced increases in LDL cholesterol and atherogenesis, these animals have been preferred as model systems of human LP metabolism. A review of the *in vivo* animal model systems has recently been published.⁵

Mammals with predominately HDL include dogs, cats, horses, ruminants, rats, mice, possibly the chimpanzee, and most other mammals.^{4,7} These animals, by contrast, are more resistant to LDL cholesterol elevations and associated atherogenesis. Nonetheless, comparative studies have discovered new concepts of lipoprotein metabolism that have been equally helpful in efforts to understand the relationship of lipoprotein metabolism to cardiovascular disease. Some of these comparative differences provide the focus for the present discussion.

TABLE 2
High Density Lipoprotein Characteristics
of Selected Species

Lipoprotein	Species	Hydrated Density g/ml	Electrophor Mobility	Major Apo-Proteins	Function
HDL ₁ (HDL ₂)	Dog	1.025-1.100	α_2	E, A, C	reverse chol. transport
	Rodent	"	"	"	"
HDL ₂	Human	1.063-1.125	α	A-1, A-II, E, C	reverse chol. transport
	Dog	1.063-1.10	α_1	A, C, E	"
	Cat	1.063-1.10	α	A-I, E, C	"
	Horse	1.063-1.18	α	A-I, A-II, E, C	"
	Rodent	1.063-1.125	α	A-I, A-II, E, C	"
	Rabbit	1.063-1.125	α	A-I, A-II, E, C	"
	Squirrel	1.063-1.125	α	A-I, A-II, E, C	"
	Monkey	1.063-1.125	α	A-I, A-II, E, C	"
	Rhesus	1.063-1.125	α	A-I, A-II, E, C	"
HDL ₃	Human	1.125-1.185	α	A-I, A-II, E, C	"
	Dog	1.10-1.21	α_1	A, C	"
	Cat	1.10-1.21	α	A, C	"
	Horse	1.118-1.21	α	?	"
	Rodent	1.125-1.21	α	A-I, A-II, E, C	"
	Rabbit	1.125-1.185	α	A-I, A-II, E, C	"
	Squirrel	1.125-1.21	α	A-I, A-II, E, C	"
	Monkey	1.125-1.21	α	A-I, A-II, E, C	"
	Rhesus	1.125-1.21	α	A-I, A-II, E, C	"
	Monkey	1.125-1.21	α	A-I, A-II, E, C	"

LOW DENSITY LIPOPROTEIN FORMATION AND METABOLISM

After assembly in the intestinal mucosal cell, chylomicrons containing cholesterol, TG, and the apo-B₄₈ peptide are secreted into lacteals. From the lymphatic system they enter the bloodstream via the thoracic duct. Transfer of apo-C and apo-E peptides from HDL occurs both in the interstitial fluid of the intestinal villus and the blood which helps direct subsequent metabolic events (Fig. 2). Peripheral tissues are perfused with these particles and their TG component is hydrolyzed via lipoprotein lipase (LPL). Important to this process is the apo-C-II peptide acquired from HDL which is a co-factor for LPL activity. The net result: as TG hydrolysis continues, free fatty acid uptake by the respective tissues occurs with the formation of cholesterol-rich chylomicron remnants containing excess surface phospholipid and apo-C peptides. These surface materials transfer to other circulating lipoproteins, yielding a cholesterol-rich, apo-E containing remnant particle. Hepatocyte remnant recep-

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tors, which recognize apo-E, rapidly remove these remnants, assuring delivery of intestinal cholesterol directly to the liver (Fig. 2). This cholesterol can be stored as its fatty acid ester, excreted either as such or as bile acids, and/or re-secreted as VLDL.

Transport and tissue distribution of endogenously synthesized TG is a well-known function of VLDL prevailing in the post-absorptive state. This LP fraction also carries a significant cholesterol load, some of which may ultimately become LDL. A precursor-product relationship between VLDL and LDL thus exists. This sequence of events begins with hepatic synthesis and secretion of VLDL containing the apo-B₁₀₀ peptide into the circulation. Once secreted, the LP particles can acquire apo-C and Apo-E peptides from HDL similar to the post-prandial chylomicron. Lipoprotein lipase mediated hydrolysis occurs at the capillary endothelium of tissues supplying fatty acids for cellular needs and VLDL-remnants are formed. In many species, most of the VLDL remnants are taken up by the liver via receptor or non-receptor mediated mechanisms (Fig. 2).⁸

In humans, a large VLDL fraction normally undergoes further processing to form LDL. Small VLDL form smaller remnants and these are more likely to become LDL than larger remnants. The larger remnants are more likely to be removed by the liver.^{9,10} The sizes and densities of the VLDL and remnant populations overlap, but remnants appear to have reduced electrophoretic mobility possibly due to loss of excess surface apo-C peptides.¹¹ Remnants with $d = 1.006-1.019$ g/ml are frequently referred to as IDL but these particles may not be qualitatively different from remnants of lower density.

The formation of LDL from VLDL is accompanied by further loss of TG, phospholipids, apo-C, and apo-E. Another lipase, hepatic lipase, participates in this additional delipidation process and apo-E transfers to HDL. In this manner, formation of a cholesteryl ester-rich LDL particle containing a single apo-B₁₀₀ molecule results. Cell surface receptors of numerous tissues can bind both apo-B and apo-E peptides and participate in the final delivery of the LP and its cholesterol payload to meet individual cellular needs (Fig. 2).

The LDL, containing apo-B₁₀₀, are removed from the circulation by the apo-B,E receptor. Similarly, VLDL remnants are efficiently taken up via apo-E binding of this receptor.⁸ It should be noted that receptor-mediated LDL uptake appears to be less efficient than remnant uptake allowing for a more gradual access of LDL to other tissues' LDL receptors. In this way, distribution of LDL cholesterol occurs to a variety of tissues.

Regarding hepatic remnant uptake, loss of apo-C peptides and other surface components of VLDL during TG hydrolysis may allow better accessibility of apo-E recep-

tor binding sites. When VLDL remnants are taken up rapidly by the LDL receptor, very little LDL forms. Possible explanations include efficiency of hepatic uptake of VLDL remnants, high LDL receptor activity, and VLDL remnant size and/or apo-E content. Larger VLDL remnants may contain more apo-E molecules which may result in tighter binding and internalization. Normal rat VLDL with its full complement of apo-C peptides bind poorly to LDL receptors, while their remnants bind tightly and are taken up rapidly. Large VLDL from normal humans, however, are not metabolized by the LDL receptor.¹² They will bind only if their apo-E is in an accessible conformation. In hypertriglyceridemic subjects, such accessibility appears to be acquired, possibly due to longer residence times of large, TG-rich VLDL in the circulation. If so, these VLDL may also be ultimately converted to LDL carrying with them the potential for oxidative modification and associated atherogenic risk.^{13,14}

LDL FUNCTION: CHOLESTEROL TRANSPORT?

Although serum TG concentrations are markedly increased during the post-prandial period, of all the organic constituents in blood during the normal, post-absorptive state, only glucose is present in molar concentrations higher than cholesterol.¹⁵ In humans, most of this cholesterol is ultimately associated with LDL which, when elevated, imparts atherogenic risk. Reasons for such relatively high steady-state concentrations of plasma LDL cholesterol, even in humans not at risk for atherosclerosis and compared with other mammals, are unclear.

The most commonly accepted explanation is that LDL is needed to provide extrahepatic cells with either endogenous or exogenous cholesterol that has been initially packaged in the liver. After all, the accepted role of LDL is to transport cholesterol and LDL receptors help perform this function. HDL mammals, however, tend to be atherosclerosis-resistant and transport most of their plasma cholesterol in HDL. When hypercholesterolemia occurs, their HDL fractions become cholesterol enriched rather than the LDL fractions. Why do these differences exist? Is LP cholesterol really a delivery vehicle for cholesterol in LDL mammals or is LDL cholesterol simply a part of the LP remnant left behind after TG delivery? Do differences in TG metabolism of species with low LDL exist such that only small amounts of VLDL are converted to LDL? Alternatively, more efficient mechanisms of LDL utilization might exist. If so, why do dogs lose their ability to express hepatic LDL receptor (apo-B,E) binding upon maturation yet maintain low plasma LDL levels?

Recently, speculation regarding the functionality of LDL as a cholesterol transport vehicle has raised a number of potentially significant points against this role for LDL.

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First, the liver secretes most of its cholesterol as VLDL and not LDL. Second, extrahepatic, de novo, cholesterol synthesis is appreciable in most species, including humans.¹⁶ In fact, LDL mammals have higher contributions of extrahepatic synthesis compared to hepatic synthesis as a percentage of total body synthesis; HDL mammals are just the opposite. Third, studies of a number of species indicate that 60 to 80% of the LP-bound cholesterol secreted by the liver is recirculated back to the liver and not taken up by extrahepatic tissues.¹⁶ Thus the tissue demands for cholesterol appear to be met by the tissues themselves and are thus less dependent on LP delivery of this lipid.

Conversely, both endogenous (chylomicron) and exogenous (VLDL) TG need to be transported as lipoprotein particles so that their fatty acid energy can be utilized by tissues. This transport requires, among other things, a package containing both free and esterified cholesterol. Thus, as TG depletion and fatty acid tissue delivery occurs, the need to eventually dispose of or re-utilize the delivery system becomes necessary. While many tissues can utilize cholesterol, most of them synthesize enough of their own supply de novo thereby necessitating the ultimate return of LP cholesterol to the liver. In liver tissues, mechanisms are in place to eliminate large quantities of cholesterol from the body. The precise way in which this is done varies among species and provides some understanding of differences seen in various mammalian lipoprotein profiles. Speed and efficiency in performing this task may be both a determinant of steady state plasma cholesterol concentrations as well as an index of resistance to atherogenesis. Thus, the extent to which the animal body compensates for high dietary fat intakes limits its production of VLDL, binds cholesterol-rich LPs or remnants, and minimizes LDL accumulation which helps define that animal's atherogenic risk. Dogs and other resistant animals seem to be particularly well adapted in this regard.

One intriguing explanation for adaptation of dogs to maintain low LDL levels even in the face of elevated serum cholesterol involves recent evidence on the ability of canine liver to undergo apolipoprotein B messenger RNA (mRNA) editing; this results in the production of both apo-B₄₈ and apo-B₁₀₀. Apo-B₄₈ has, heretofore, been thought to be exclusively intestinally synthesized in most mammals. The process of mRNA editing refers to the alteration of an RNA's informational capacity other than by known processes such as RNA splicing, 5' end formation, and 3' endonucleotide cleavage and polyadenylation. A discussion of this type of modification is beyond the scope of this review but details are available.^{17,18} Chylomicron remnants which typically contain apo-B₄₈ and apo-E tend to be rapidly cleared after TG delivery. TG-rich lipopro-

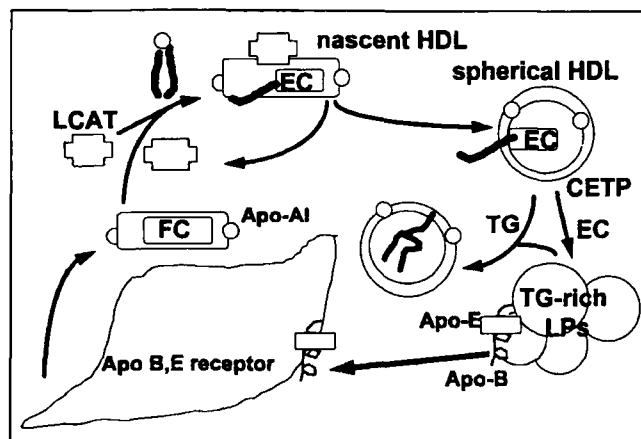


FIG. 3 — Lecithin:cholesterol acyl transferase activity and reverse cholesterol transport: Small, discoidal HDL (nascent HDL) secreted by the liver can pick up unesterified cholesterol from peripheral cells. This cholesterol is esterified via LCAT action using discoidal HDL lecithin as fatty acid source. Apo-AI participates as a co-factor in the reaction. Spherical, cholesteryl ester-rich HDL result. In the presence of cholesteryl ester transfer protein (CETP) triglyceride can be exchanged for cholesteryl ester between low and high density fractions. The apo-B,E receptor then modulates cholesterol ester-rich particle uptake completing the reverse cholesterol transport process. Dogs, horses, and mice have low CETP. Thus mechanisms to return cholesterol via HDL mediated events likely exist in these animals (see text for details).

teins containing apo-B₁₀₀ (i.e., VLDL), however, may have longer residence times in the circulation and thus are more likely to serve as LDL precursors. It is of interest that dogs, horses, rats, and mice (all HDL mammals) appear to be able to produce either apo-B peptide from a single apo-B₁₀₀ gene via mRNA editing.¹⁸ In the intestine such editing provides a lipoprotein system for the absorption of dietary lipid and rapid hepatic uptake. When also synthesized in the liver, the edited apo-B₄₈ are secreted as part of VLDL providing a mechanism whereby endogenous TG packaging and transport can be handled rapidly and efficiently. The net result is minimal accumulation of lipid rich particles containing apo-B₁₀₀ in the circulation. Other adaptational responses of dogs may also contribute to lower atherogenic risk and will be discussed under HDL metabolism and reverse cholesterol transport below.

HDL AND CHOLESTEROL ESTERIFICATION

The liver is the site of nascent HDL synthesis.¹⁹ The major proteins of HDL, apoA-I and apoA-II, appear to be synthesized in the liver although apoA-I also originates intestinally. In the absence of an active lecithin:cholesterol acyl transferase (LCAT) enzyme, the newly formed HDL appear as discoidal particles rather than spherical. These particles contain lecithin, apoA-1, and possibly free cholesterol. The LCAT enzyme, synthesized in the liver, is

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active in plasma, and responsible for the formation of cholesteryl esters (CE) utilizing phosphatidyl choline fatty acid and free cholesterol as substrates. Esterification of cholesterol by LCAT results in spherical HDL particles containing appreciable amounts of CE. Evidence exists that "discoidal" HDL pass through a LP intermediate with pre- β electrophoretic mobility on their way to becoming spherical (Fig. 3).²⁰

Reverse Cholesterol Transport CHOLESTERYL ESTERTRANSFER

It has been noted that plasma HDL performs a peptide donor function such that apo-C and apo-E peptides can be transferred to VLDL facilitating triglyceride hydrolysis, remnant metabolism, and tissue lipid delivery (Fig. 1). This sequence of events might be termed "forward cholesterol transport". A second, equally important, role for HDL is its involvement in the return of cholesterol from peripheral cells to the liver; a process known as "reverse cholesterol transport." The balance between the forward and reverse pathways determines net plasma cholesterol concentrations, the residence times of the various LP fractions, and the physical and metabolic state of that cholesterol in the circulation.

Overall, reverse transport of cholesterol in humans can be described by the following series of reactions: Small, discoidal HDL initially participate in the desorption of cell membrane free cholesterol from peripheral tissues. This cholesterol is then esterified by LCAT and the cholesterol esters (CE) become interiorized into the HDL core. Next, CE transfer occurs in which a cholesteryl ester transfer protein (CETP) mediates the transfer of the CE to other lipoprotein particles (i.e., VLDL/LDL). This process occurs in exchange for triglyceride (Fig. 3). Finally, a step in which the CE is interiorized by the liver either by apo-B₁₀₀ or apo-E receptor mediated events completes the reaction sequence. Recently evidence for the selective uptake of HDL-CE without necessitating uptake of the lipoprotein protein has been obtained.^{21,24} Both mechanisms are believed to participate in hepatic uptake.

Cholesterol Efflux Out of Cell Membranes

The major force guiding cholesterol out of most cells is likely to be its LCAT catalyzed esterification in plasma which utilizes cell membrane derived free cholesterol as a major substrate.²⁰ Both receptor-mediated and diffusion-controlled desorption methods have been proposed as mechanisms. Evidence exists to indicate that a pre- β migrating, small apoA-I containing HDL (nascent HDL) may be a particularly effective acceptor of this cell-derived cholesterol and that, in addition to its ability to esterify free cholesterol, LCAT may also be specifically involved in this transport process. Such specificity would

increase the effectiveness of reverse cholesterol transport especially since most of the regulation of LCAT occurs post-translationally and is activated post-prandially or by dietary fat.^{20,22,25}

Cholesteryl Ester Transport Protein Activities and Atherosclerosis Resistance ANIMAL MODELS

While most species examined to date appear to have active LCAT,^{23,26} differences exist regarding the expression of cholesteryl ester transfer protein (CETP) activity among mammals.^{3,27,28} This difference may help explain the existence of LDL vs. HDL mammals because, as noted previously, CETP is involved in the transfer of cholesteryl ester from HDL to LDL in exchange for TG. Atherosclerosis resistant species which have no or low CETP activities include mouse, rat, sheep, cow, pig, dog, and possibly cat.^{27,28} In the absence of CE transfer, HDL remain enriched in CE and apo-E resulting in a larger and less dense particle. In dogs this particle has a hydrated density of 1.025 to 1.100 g/ml which overlaps with that of LDL. It has been designated HDL₁ or HDL_c, has α_2 migration on electrophoresis,²⁹ and can be induced by high dietary fat intakes.³⁰⁻³² In this case, reverse cholesterol transport is likely completed via hepatic HDL cholesterol uptake, rather than CE-rich VLDL/LDL uptake.

In atherosclerosis resistant dogs it is noteworthy to point out that puppies appear to have both apo-B,E and apo-E receptor activities on liver membranes that can be down regulated with cholesterol feeding. Upon maturation, however, expression of this receptor activity is lost.³⁰ Only apo-E receptor activities remain and these can bind canine cholesterol-rich HDL. Similarly, in adult pigs only apo-E receptors have been found while membranes from fetal swine livers expressed both types. Consistent with these findings, in puppies LDL fractional clearance was 50% faster compared to adult dogs.³⁰ Physiologically, the possibility exists that a generalized increase in apo-B,E receptor activity is needed in puppies to help scavenge LDL cholesterol after supplying high triglyceride-energy containing lipoproteins during rapid growth. Induction of the apo-B,E receptor with prolonged (3.5 days) fasting of adult dogs also supports this possibility. Taken together, these data indicate that both LDL (human and pig) and HDL mammals (dog) have apo-B,E and apo-E receptors in common which may be expressed under certain physiologic and/or dietary conditions. These data suggest a central role of apo-E receptors and apo-E containing lipoproteins in mediating LP clearance.

The production of arterial lesions in dogs occurs only if plasma cholesterol can be elevated above about 750 mg/dl. In order to accomplish such a marked increase, induction of hypothyroidism and diets containing fat, cho-

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lesterol, and bile salts must be fed. Alternatively, feeding euthyroid dogs high cholesterol, saturated fat diets (42 energy % fat) for 1 year will also induce hypercholesterolemia.³⁰ Even under these conditions, while two- to five-fold elevations in serum cholesterol occurred in many dogs, TG concentrations remain normal and lesions were absent. Only when hypercholesterolemia exceeded 750 mg/dl did lesions and hypertriglyceridemia result. We have found two- to threefold elevations in serum cholesterol 3 weeks after normal and endurance-trained dogs had been fed diets containing from 55 to 63 energy % as fat.^{31,32} Alterations in lipoprotein distributions included significant increases in α_2 -migrating LPs (i.e., HDL₁ or HDL₂) up to 750 mg/dl cholesterol. Only when this value was exceeded were both α_2 - and β -migrating (LDL) lipoproteins observed.³⁰ The α_2 LPs are CE-rich and contain appreciable amounts of apo-E. Presumably, they are formed by LCAT-mediated esterification and function to help return the excess cholesterol to the liver. The lack of CETP activity of dog serum may, therefore, help impart resistance to atherogenesis since CE enrichment of LDL does not occur.

HUMANS

Identification of human individuals that completely lack plasma CETP due to a common mutation that prevents normal processing of RNA has been reviewed.³³ Homozygotes have high plasma cholesterol, most of which is in HDL. Heterozygotes have plasma cholesterol and HDL levels within either the normal or high-normal range. ApoA-I levels are increased twofold in homozygotes and above normal in heterozygotes. Analysis of HDL subclasses shows relatively normal HDL₃ but markedly increased HDL₂. These latter particles are larger in size with hydrated densities in the typical LDL region (i.e., 1.05-1.063 g/ml) and are enriched in apo-E.³³ Humans with complete absence of CETP do not appear to suffer any deleterious consequences and seem to benefit by virtue of an increased lifespan and decreased incidence of coronary artery disease.^{34,35} While many of these changes appear similar to HDL metabolism in dogs, it remains to be determined what overall effect lack of CETP has on LDL metabolism in humans.

Lipoprotein Metabolism in Transgenic Mice

As advances in molecular biology of lipoproteins occur, numerous lipoprotein apopeptide, enzyme protein, and receptor genes have been isolated, sequenced, mapped, and used to help identify mutations associated with coronary heart disease. Transgenic mice of the following types have been developed to various extents: ApoA-I, apoA-II, ApoA-IV, apo-B, apo-C, apo-E, apo-Lp(a), CETP, LPL, and LDL receptor. These have recently

been reviewed.³⁶ These modified mice species will continue to augment existing animal models of human LP metabolism. Some of these models are associated with increased susceptibility to atherosclerosis and include those with increased LDL-cholesterol, low HDL cholesterol with and without hypertriglyceridemia, and increased chylomicron remnants.³⁶ An apo-E deficient mouse has also been recently produced which subsequently developed lesions resembling human atherosclerosis when fed a laboratory chow diet.³⁷ Use of murine models that have been genetically transformed from a species that is highly resistant to atherosclerosis to one that is highly susceptible will assist future studies of diets, genetics, and other factors that influence the relationship between lipoprotein metabolism and atherogenesis. ■

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Abstracts

Glanzmann's Thrombasthenia in a Great Pyrenees

An 8-month-old, female, Great Pyrenees dog, with chronic epistaxis and a history of gingival bleeding during shedding of deciduous teeth, was referred for platelet evaluation. Von Willebrand factor antigen levels were within the reference range. Platelet number and mean platelet volume were normal; platelet morphology was normal at the light and electron microscopy level. Platelet aggregation responses to ADP, collagen, PAF, and thrombin were markedly reduced, although shape change responses were normal. Clot retraction was markedly impaired. Monoclonal antibody (MoAb) Y2/51, a murine anti-human platelet $\beta 3$ antibody which cross-reacts with canine platelet $\beta 3$, and MoAb 5G11, a murine anti-canine platelet $\alpha IIb\beta 3$ antibody, bound minimally to affected dog platelets as demonstrated by flow cytometry. Binding of MoAb Y2/51 was not detectable by immunoblot technique. A reduction in glycoproteins αIIb and $\beta 3$ was demonstrated by two dimensional protein electrophoresis. This is the first reported case of Type I Glanzmann's thrombasthenia in the dog that closely resembles the clinical syndrome and the platelet morphology described in Type I Glanzmann's thrombasthenia of human beings.

Abstracted from: Boudreaux M, Kvam K, Dillon R, et al: Glanzmann's Thrombasthenia in a Great Pyrenees. Auburn University, Auburn, AL 36849

Ultrastructural Features of Feline Eosinophils

Blood, bone marrow, and tissues from healthy cats and cats with eosinophilic diseases were examined by electron microscopy, to define features of granule development in eosinophils and the morphology of activated eosinophils. Specific granule formation is a multi-step process beginning in early precursors and continuing in mature cells. Vesiculotubular structures appear to play a role in granule formation and degranulation. Feline eosinophil granules have unique ultrastructural characteristics: cores are layered and can be multiple, hollow, or peripheralized and have a disorganized or fragmented appearance in early cells. Morphologic features of activation, however, are similar to those of human eosinophils and include piecemeal degranulation, decreased numbers of specific granules, and increased numbers of vesiculotubular structures and lipid bodies. One difference is that neither Charcot-Leyden crystals nor an increase in primary granules has been found. Peripheral blood eosinophils from cats with eosinophilic inflammation did not have morphologic evidence of activation. Morphologic changes in eosinophils may be more specific indicators of activation than other changes, such as hypodensity. Investigations into what mediates these changes should lead to a greater understanding of eosinophil biology and how eosinophilic inflammation, which can be detrimental to the host, can be controlled.

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Preclinical Evaluation of Single-cell Oils that are Highly Enriched with Arachidonic Acid and Docosahexaenoic Acid

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Abstract—Arachidonic acid (ARA) and docosahexaenoic acid (DHA) are important in human brain and retina development, and there is growing evidence showing the importance of these fatty acids in infant nutrition. Triglyceride oils, highly enriched in ARA (ARASCO®) and DHA (DHASCO®), were evaluated using very high dose acute (20 g/kg) and 4-wk subchronic gavage feedings in weanling Sprague-Dawley rats. The combination of these oils, Formulaid®, was also tested in the 4-wk subchronic study. ARASCO, DHASCO and Formulaid were found to have a no-observable-adverse-effect level of more than 2.5 g/kg/day, 1.25 g/kg/day and 3.75 g/kg/day, respectively. This represents a 50-fold safety margin over the intended use of Formulaid in infant formula. Survival, clinical signs, body weight gain, food consumption, haematology, clinical chemistry and histopathological evaluations failed to show any significant differences in animals administered ARASCO, DHASCO or Formulaid compared with that in control animals administered equal amounts of high oleic sunflower oil. The bioavailability of ARASCO, DHASCO and Formulaid was verified by increases in DHA and ARA levels in heart and liver tissues in these animals. Because these oils are enriched in only a single bioactive fatty acid, and they have been shown to be safe, they may offer a new source of these fatty acids in speciality foods such as infant formula. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Arachidonic acid (ARA) and docosahexaenoic acid (DHA) are long-chain polyunsaturated fatty acids (LC-PUFAs). ARA is a 20-carbon fatty acid with four double bonds, the first of which is located at the sixth carbon atom from the methyl terminus (ω -6 fatty acid). DHA is a 22-carbon fatty acid with six double bonds, the first of which is located at the third carbon atom from the methyl terminus (ω -3 fatty acid). ARA and DHA are the predominant ω -6 and ω -3 fatty acids found in the human brain, and DHA is an important structural component of neurological and retinal tissues (Salem *et al.*, 1986). Although these fatty acids make up a significant portion of the lipids in neurological tissues, they are not efficiently synthesized by the body, especially in infants, but are obtained directly from the diet (Bourre *et al.*, 1990; Crawford, 1993).

The most rapid period of human brain growth occurs during the last trimester *in utero* and during the first 12 months of postnatal life. During this period, the infant acquires ARA and DHA from the mother by placental transfer or from breast milk. Women on a Western diet have breast milk which typically contains 0.4% of the total lipid as ARA and 0.2% as DHA. Infants born prematurely can no longer obtain ARA and DHA from the placenta and are at a greater risk for neurological problems such as learning disabilities and social/behavioural problems (Hack *et al.*, 1992; Hoy *et al.*, 1992; Lanting *et al.*, 1994; Saigal *et al.*, 1991; Smedler *et al.*, 1992). Studies have also shown that children who were breast fed as infants (i.e. with a dietary source of DHA) score higher on cognitive tests and demonstrate more rapid retinal development than children who were formula-fed as infants (i.e. no dietary source of DHA) (Anderson *et al.*, 1990; Gibson *et al.*, 1993; Lucas *et al.*, 1992; Makrides *et al.*, 1993 and 1995; Uauy *et al.*, 1990 and 1993). Consequently, the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN), the British Nutrition Foundation (BNF), the WHO/FAO Expert Committee on Fats and Oils in Human Nutrition and the International Society for the Study of Fatty Acids and Lipids (ISSFAL) have recommended that

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Abbreviations: ALA = α -linolenic acid; ANOVA = analysis of variance; ARA = arachidonic acid; BNF = British Nutrition Foundation; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; ESPGAN = European Society of Pediatric Gastroenterology and Nutrition; GC = gas chromatography; GLA = γ -linolenic acid; ISSFAL = International Society for the Study of Fatty Acids and Lipids; LC-PUFA = long-chain polyunsaturated fatty acid.

these essential fatty acids be included in preterm infant formula. The BNF and WHO/FAO have also recommended that they be included in term infant formula (British Nutrition Foundation, 1992; ESPGAN Committee on Nutrition, 1991; FAO/WHO Expert Committee, 1994; ISSFAL Board of Directors, 1994).

Clinical studies using fish oil to provide DHA in infant formula resulted in a depression of growth rate of the infants (Carlson, 1991; Carlson *et al.*, 1992). This effect was the likely result of the significant drop in ARA levels caused by eicosapentaenoic acid (EPA) in the fish oil. In addition, the positional specificity of DHA within the triacylglycerol in fish oil is different from that in human milk (Christenson and Høelmer, 1993). Egg yolk has also been added to infant formula to provide ARA and DHA, but the ARA and DHA in egg yolk are in the form of phospholipid, unlike the triacylglycerol form found in human milk. To match the levels of ARA and DHA in human milk, quantities of egg yolk phospholipid would have to be added to the formula that are far in excess of recommended upper limits for infant formula (UK Ministry of Agriculture Fisheries and Food, 1991). In addition to the excess amount of phospholipid compared with that in human milk, egg yolk also adds a level of cholesterol far in excess of that found in human milk.

Given the results of these studies and the need for a dietary source of these fatty acids which more closely matches human milk, an infant formula additive that provides both ARA and DHA with no other LC-PUFA has been developed (i.e. Formulaid®). Traditionally, fish oils have been a source of LC-PUFA, but fish derive their LC-PUFA from the phytoplankton that they eat. This algal source for production of DHA (DHASCO®) was used in this study. Concurrently, a single-cell source of ARA (ARASCO®) was developed based on oil extracted from a soil fungus which is commonly found in association with many root crops. Formulaid is the combination of ARASCO, a single-cell oil containing 40% ARA, and DHASCO, a single-cell oil containing 40% DHA. ARASCO and DHASCO are in the triacylglycerol form as in human milk, and the positional specificity of DHA within the triacylglycerol is preferentially on sn1 and sn3, also similar to that of human milk (Christensen and Høelmer, 1993; Kyle *et al.*, 1992).

Both ARASCO and DHASCO represent dietary supplements that can elevate the levels of ARA and

DHA in conventional foods (specifically infant formula). The results of acute toxicity studies of ARASCO and DHASCO, and a 4-wk oral gavage toxicity study of ARASCO, DHASCO and Formulaid supplementation in rats are reported here.

MATERIALS AND METHODS

Test materials

The materials tested in the acute oral toxicity study and the 4-wk oral gavage toxicity study were ARASCO (lot nos AD003 and A011-DS-2, respectively), a single-cell triglyceride oil produced by *Mortierella alpina*, and DHASCO (lot no. DD004-WS), a single-cell triglyceride oil produced by *Cryptocodinium cohnii*. Both *M. alpina* and *C. cohnii* were grown in 80000-litre fermenters under controlled production procedures, and the oils were extracted from the biomass, refined, bleached and deodorized according to standard operating procedures (Kyle, 1994; Kyle *et al.*, 1995). The *trans*-fatty acid content of each of the oils was less than 3% as determined by infra-red spectroscopy following the American Oil Chemists' Society method number Cd14-61 (Firestone, 1993). Antioxidants, ascorbyl palmitate (0.025%, w/w) and tocopherol (0.005%, w/w) were added to the final products. Formulaid (lot no. F011-DS-2), also tested in the 4-wk oral gavage study, was produced by mixing ARASCO and DHASCO so that the ratio of ARA to DHA was 2:1. The fatty acid profiles of the ARASCO, DHASCO and Formulaid lots which were used in the acute oral toxicity and 4-wk oral gavage toxicity studies are shown in Table 1.

Acute oral toxicity of ARASCO and DHASCO

The following acute oral toxicity study was performed at Corning Hazleton, Inc., Madison, WI, USA following the direction of NIH publication no. 86-23 (revised 1985, US DHHS).

Animals. 10 male and 10 female, approximately 8-wk-old albino rats (CrI: CD*Br) were obtained from Charles River Laboratories, Inc. (Portage, MI, USA). The animals were acclimatized for 7 days and housed in groups (five/cage) according to sex. They were kept in a temperature- and humidity-controlled environment with a 12-hr light/dark cycle. Rodent chow no. 5001 (Purina Mills, Inc.) and water were provided *ad lib.*, except overnight before test material administration when food was withheld. At

Table 1. Fatty acid composition of ARASCO, DHASCO and Formulaid used in toxicity studies

	Fatty acid composition (% of total fatty acids)											
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	24:0	22:6
ARASCO	0	0.4	7.8	0	10.5	14.0	7.1	4.0	4.3	48.5	1.3	0
DHASCO	4.4	19.4	18.1	1.8	0.4	15.4	0.6	0	0	0	0	38.4
Formulaid	1.5	7.3	12.2	0.7	7.1	15.0	4.7	1.9	1.3	30.9	0.6	14.1

Table 2. Experimental design of 4-wk oral gavage toxicity study of ARASCO, DHASCO and Formulaid

Group	Dose level				No. of animals	
	ARASCO (mg/kg)	DHASCO (mg/kg)	Formulaid (mg/kg)	High oleic sunflower oil (mg/kg)	Males	Females
1 (control)	—	—	—	3750	10	10
2	50	—	—	3700	5	5
3	1000	—	—	2750	5	5
4	2500	—	—	1250	10	10
5	—	25	—	3725	5	5
6	—	500	—	3250	5	5
7	—	1250	—	2500	10	10
8	—	—	1500	2250	5	5
9	—	—	3750	—	10	10

initiation, the animals weighed 214–252 g for the ARASCO group and 202–260 g for the DHASCO group.

Test material administration. Five animals/sex were given a single dose of either undiluted ARASCO or DHASCO by oral gavage. ARASCO was given at 21.74 ml/kg and DHASCO was given at 22.73 ml/kg. On the basis of average bulk densities of 0.92 g/ml and 0.88 g/ml, respectively, both doses corresponded to 20 g oil/kg body weight.

Observations. The animals were observed for clinical signs and mortality at 1, 2.5 and 4 hr after dosing, and then once daily for 14 days.

Body weight. Body weights were determined before dosing, and on days 7 and 14.

Pathology. On day 14 after dosing the animals were killed and an autopsy was performed.

4-Wk oral gavage toxicity of ARASCO, DHASCO and Formulaid

The following 4-wk oral gavage toxicity study was performed at Corning Hazelton Inc., (Madison, WI, USA).

Animals. 71 male and 71 female, 4–5-wk-old rats (CrI: CD*(SD)BR VAF/Plus*) were obtained from Charles River Laboratories, Inc. (Portage, MI, USA) and acclimatized for 11 days before initiation of treatment. The animals were housed individually in stainless-steel, screen-bottomed cages and kept in an environment maintained at 19–25°C, 50% humidity and a 12-hr light/dark cycle. Certified rodent chow no. 5002 meal from PMI® Feeds, Inc. was provided *ad lib.*, except when the animals were fasted. Water was also provided *ad lib.* After an examination to determine suitability for study, animals of each sex were ranked by weight. Animals with body weights exceeding $\pm$ two standard deviations of the mean weights were excluded. The selected animals were assigned a random number and allocated to groups according to the relative rank of the random number. At initiation of the test material administration, the rats were 5–6-wk old, the males weighing from 130 g to 186 g, and the females weighing from 138 g to 176 g. The experimental design is outlined in Table 2.

Test material preparation and administration. Test material doses of 50, 1000 and 2500 mg ARASCO/kg/day, 25, 500 and 1250 mg DHASCO/kg/day and

1500 and 3750 mg Formulaid/kg/day were prepared by diluting the test materials with high oleic sunflower oil (lot no. 10213, SVO Specialty Products, Eastlake, OH, USA) so as to provide a constant total oil dose of 4.17 ml/kg/day (4.0 g/kg/day) to each animal based on the most recently recorded body weights. Each dose was prepared once, divided into daily aliquots and stored at –20°C. A sample of each mix was retained to verify the fatty acid profile by gas chromatography (GC). Daily aliquots were thawed to room temperature before use, and the test materials were administered by oral gavage once daily for 4 wk. Control animals were dosed only with high oleic sunflower oil.

Observations, body weights and food consumption. The animals were observed twice daily for mortality, and any signs of toxicity were recorded. Each animal was removed from its cage and examined at least once weekly. Body weights were determined on the first day of treatment and weekly thereafter. Food consumption was recorded weekly during treatment.

Blood sample collection. Blood (approx. 1 ml) was collected from the retro-orbital plexus before the initiation of treatment and at the end of wk 2 from five animals/sex group. The animals were fasted overnight and anaesthetized with ketamine before blood collection. At wk 5, the animals were fasted overnight, anaesthetized with sodium pentobarbital and blood was collected from the posterior vena cava. Serum was harvested and stored at –20°C for future analysis.

Haematology and clinical chemistry. Red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelet count, white blood cell count, differential blood cell count, blood cell morphology, reticulocyte count smear (made, but not examined) and prothrombin time were measured for each animal of each group at the termination of the in-life portion of the study. Although platelet aggregation was not measured in this study, clinical trial data have shown that DHASCO does not inhibit collagen-induced platelet aggregation, thromboxane production, or alter fibrinogen, factor VII levels, or serum viscosity (Conquer and Holub, 1995).

Serum samples taken at the termination of the in-life portion of the study were analysed for glucose, urea nitrogen, creatinine, total protein, albumin, globulin, total bilirubin, cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transferase, calcium, inorganic phosphorus, sodium, potassium and chloride.

Pathology. At the end of the treatment period, the rats were fasted overnight, anaesthetized with sodium pentobarbital, weighed and exsanguinated. An autopsy was performed that included examination of the: external surface of the body, all orifices, cranial cavity, external surfaces of the brain and spinal cord, nasal cavity and paranasal sinuses, and thoracic abdominal and pelvic cavities and viscera.

Several organs including adrenals, brain, heart, kidneys, liver and testes were excised and weighed (paired organs were weighed separately). Organ-to-body weight percentages and organ-to-brain weight ratios were calculated.

The following tissues were collected from each animal and preserved in 10% phosphate buffered formalin: adrenals, aorta, brain, caecum, colon, duodenum, epididymes, oesophagus, eyes, femur, heart, ileum, jejunum, kidneys, lacrimal gland, lesions, liver, lungs, lymph node, mammary gland, muscle, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skin, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid, trachea, urinary bladder and uterus. All tissues were embedded (sternum and femur were decalcified before embedding) in paraffin, sectioned, stained with haematoxylin and eosin, and initially examined microscopically from five animals/sex from the highest dose groups.

In addition to routine tissue analysis, samples of the brain, heart, liver and right testis were collected from five animals/sex from the highest dose groups. The organs were frozen in liquid nitrogen, dried under vacuum, and fatty acid methyl esters were prepared and analysed by GC using an Omegawax™ 250-fused silica capillary column (Supelco, Bellefonte, PA, USA).

Statistical analysis. Levene's test (Levene, 1960) was performed to determine whether the groups had equal variances. If the variances were not equal ($P \leq 0.05$), transformations were used to stabilize the variance, and then an analysis of variance (ANOVA) (Winer, 1971) was performed. If significant differences among the means were found, Dunnett's *t*-test (Dunnett, 1964) was used to determine which means were significantly different from that of the control. If a significant difference occurred in the highest dose groups when evaluated at the 5% two-tailed probability level, then a one-way ANOVA was used to evaluate these data. If the ANOVA was significant, a Games and Howell

(1976) modified Tukey-Kramer test was used for all possible pairwise comparisons between those groups. The tissue fatty acid results were evaluated using Student's *t*-test at the 5% two-tailed probability level.

RESULTS

Acute oral toxicity of ARASCO and DHASCO

Observations, body weights and pathology. Following gavage of ARASCO (20 g/kg) all animals experienced watery/soft stools, red-stained faces and wet/yellow-stained urogenital areas for the first few hours. Such observations were not unexpected given such a high dose of oil, and all the animals appeared normal from days 4 to 14. Gavage of DHASCO (20 g/kg) similarly resulted in animals with soft stools during the first 4 hr and dark-stained urogenital areas for 2 days. All animals in the DHASCO-treated group also appeared normal from days 3 to 14. All animals in both groups gained weight at a normal rate, and no visible lesions were present at autopsy.

4-Wk oral gavage toxicity of ARASCO, DHASCO and Formulaid

Observations, body weights and food consumption. All animals survived to the scheduled sacrifice, and there were no test material-related antemortem observations. There were no significant differences between groups in body weight gains or food consumption throughout the study (Fig. 1 and Fig. 2).

Haematology and clinical chemistry. Some females in the middle-dose ARASCO group and the high-dose DHASCO group had a significantly lower total protein value than those in the control group. Albumin was also significantly lower for females in the middle and high-dose ARASCO groups and the high-dose DHASCO group, although there was no dose-response relationship. The males were not similarly affected. High serum potassium levels were observed in several animals, including those in the control group, and were not considered to be test material related. There were no clinical pathology changes consistent with haemolysis such as lower red blood cell levels or haematocrit, or higher mean corpuscular volume, nor were there microscopic findings consistent with haemolysis in animals with or without the high potassium levels. Some individual potassium levels would have seriously affected the animals if they had been present before anaesthesia; therefore, the high potassium levels were attributed to an excessively deep plane of anaesthesia before blood collection. Although platelet aggregation was not measured in this study, clinical trial data have shown that DHASCO does not inhibit collagen-induced platelet aggregation, thromboxane production or alter fibrinogen, factor VII levels, or serum viscosity (Conquer and Holub, 1995).

There were no test material-related changes for

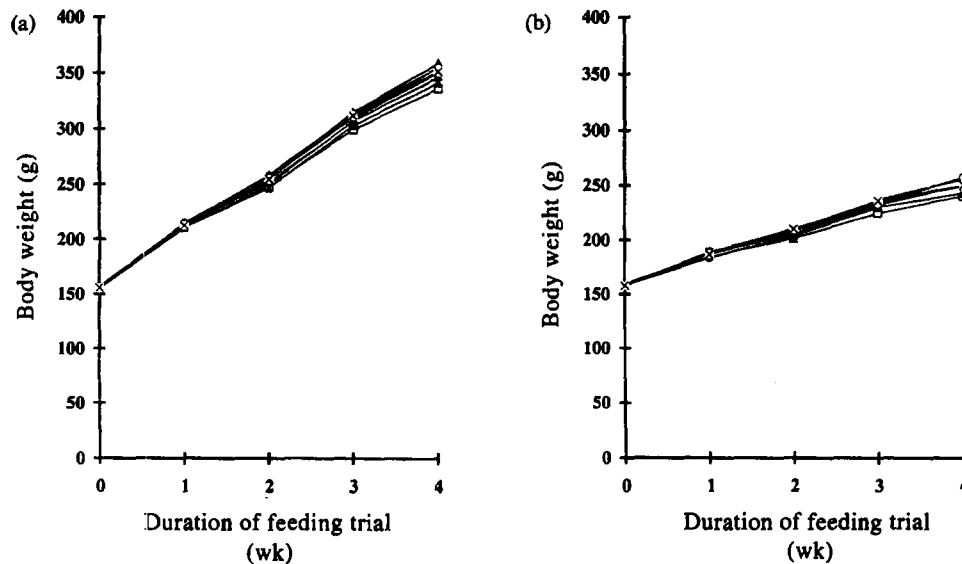


Fig. 1. Body weight gain (g/wk) in (a) male and (b) female rats after oral gavage with 3750 mg/kg/day high oleic sunflower oil (■), 50 mg/kg/day ARASCO (□), 1000 mg/kg/day ARASCO (◆), 2500 mg/kg/day ARASCO (◇), 25 mg/kg/day DHASCO (▲), 500 mg/kg/day DHASCO (△), 1250 mg/kg/day DHASCO (●), 1500 mg/kg/day Formulaid (○), or 3750 mg/kg/day Formulaid (x) for 4 wk.

terminal body weights, organ-to-body weight percentages, or organ-to-brain weight ratios. However, the liver weights of males in the high-dose Formulaid group were significantly higher than those of the control group and those of males in the high-dose DHASCO group. The liver weight changes were mild, less than 20%, and there were no abnormal microscopic findings in the livers. High levels of polyunsaturated fatty acids are known to induce oxidative stress, so stimulation of the cytochrome

P-450 system cannot be ruled out without further experimentation.

Microscopic changes included haemorrhage, macrophage infiltrate and vascular mineralization of the lungs, cysts, pelvic dilation, chronic inflammation and tubular mineralization of the kidneys, hepatocellular hypertrophy, chronic inflammation and hepatocellular necrosis of the liver, haemorrhage of the thymus, ultimobranchial cysts, ectopic thymus and chronic active inflammation of the thyroids, chronic

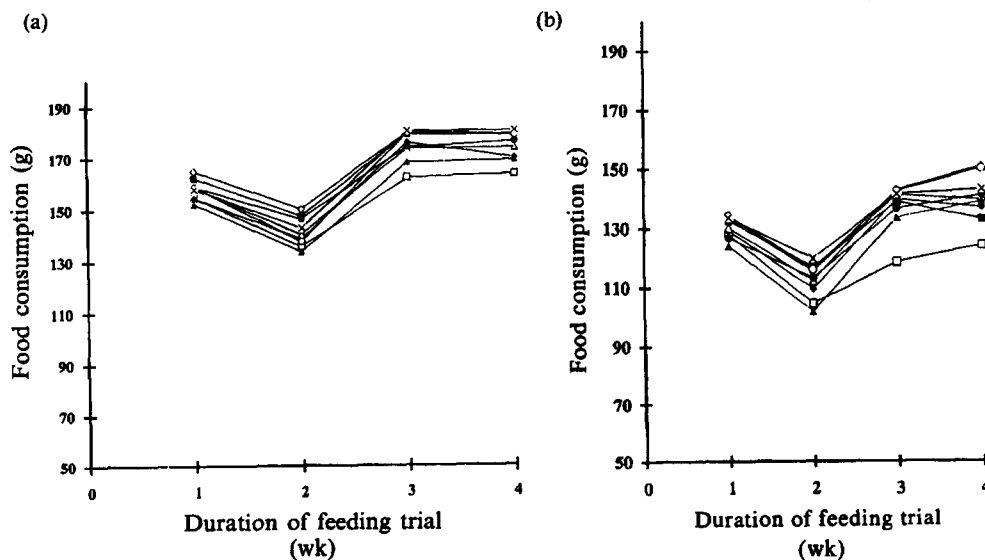


Fig. 2. Food consumption (g/wk) of (a) male and (b) female rats after oral gavage with 3750 mg/kg/day high oleic sunflower oil (■), 50 mg/kg/day ARASCO (□), 1000 mg/kg/day ARASCO (◆), 2500 mg/kg/day ARASCO (◇), 25 mg/kg/day DHASCO (▲), 500 mg/kg/day DHASCO (△), 1250 mg/kg/day DHASCO (●), 1500 mg/kg/day Formulaid (○), or 3750 mg/kg/day Formulaid (x). The reduction in food consumption in all groups during wk 2 was caused by fasting the animals for blood collection.

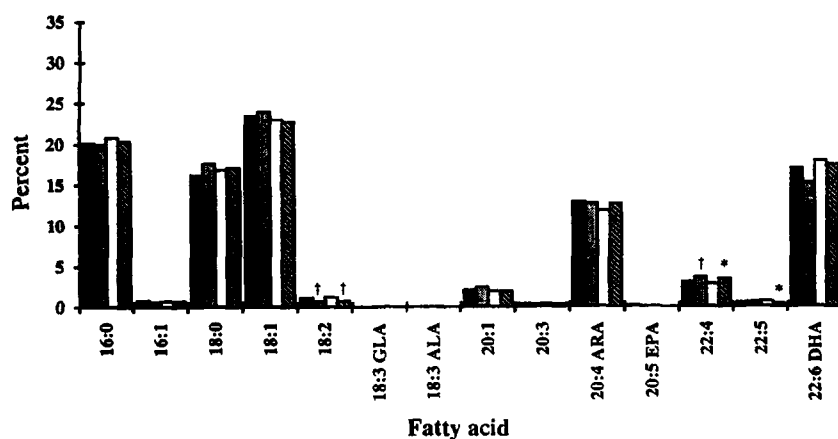


Fig. 3. Fatty acid composition of brain tissue from rats in the high-dose ARASCO (■), DHASCO (□) and Formulaid (▨) groups, and the control group (■). * $P \leq 0.05$; † $P \leq 0.001$ *v.* control values (two-tailed student's *t*-test). ALA = α -linolenic acid; GLA = γ -linolenic acid.

inflammation of the exorbital lacrimal gland, cortical vacuolation of the adrenals, dilation of the uterus, haemorrhage of the mandibular and cervical lymph nodes, and acute inflammation and ulceration of the skin. However, these changes were observed randomly between the control and treated groups and were considered not to be test material related.

Tissue fatty acid analysis. The fatty acid composition of the brain, heart, liver and testes for each of the high-dose ARASCO, DHASCO and Formulaid, and control group are presented in Figs 3–6. In general, supplementation with ARASCO led to elevation of the long-chain ω -6 fatty acids and supplementation with DHASCO led to the elevation of the long-chain ω -3 fatty acids as a percentage of total fatty acids in those tissues. These differences were more pronounced in the heart and liver (Figs 4 and 5) than in the brain and testes (Figs 3 and 6).

In brain tissue, significant changes were observed only in the minor fatty acids (18:2; 22:4 ω -6; and 22:5 ω -6), while changes in the major components did not reach statistical significance (Fig. 3). ARASCO supplementation appeared to depress 18:2 levels, while elevating 22:4 ω -6. This change was maintained even in the presence of DHASCO (i.e. in Formulaid samples).

In heart tissue, the high-dose ARASCO groups also showed a significant depression of the fatty acids 18:2 and elevation of 22:4 ω -6 as in the brain, but significant changes were also seen in a depression of 18:1 and 16:0, as well as an increase in ARA itself (Fig. 4). The high-dose DHASCO group exhibited fewer significant changes, although the DHA levels in the heart tissue increased by 56%, and ARA and its long-chain metabolites 22:4 ω -6 and 22:5 ω -6 were all significantly reduced. When both oils were added together (Formulaid) only ARA and 22:4 ω -6 returned to levels found in the control tissues. This

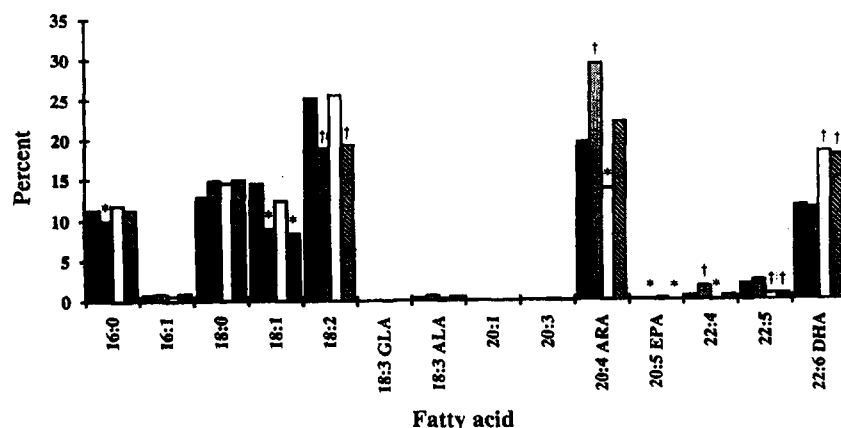


Fig. 4. Fatty acid composition of heart tissue from rats in the high-dose ARASCO (■), DHASCO (□) and Formulaid (▨) groups, and the control group (■). * $P \leq 0.05$; † $P \leq 0.001$ *v.* control values (two-tailed Student's *t*-test). ALA = α -linolenic acid; GLA = γ -linolenic acid.

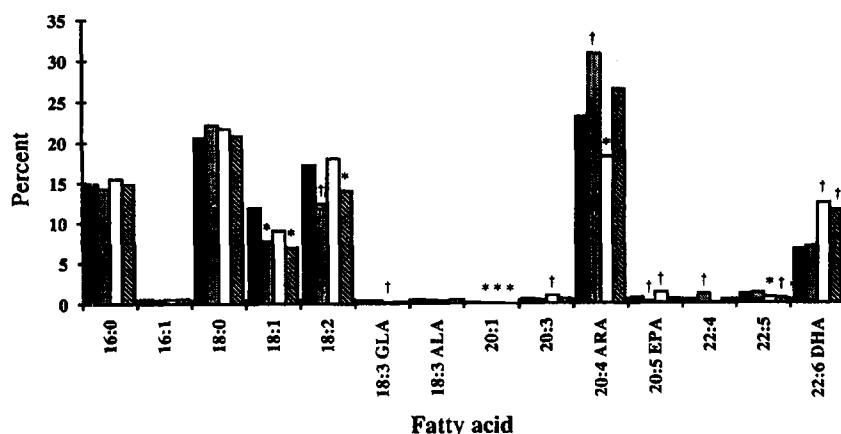


Fig. 5. Fatty acid composition of liver tissue from rats in the high dose ARASCO (■), DHASCO (□) and Formulaid (▨) groups, and the control group (■). * $P \leq 0.05$; † $P \leq 0.001$ v. control values (two-tailed Student's *t*-test). ALA = α -linolenic acid; GLA = γ -linolenic acid.

suggests that DHA has little effect on the suppression of 18:1 and 18:2 by ARASCO, and that ARA has little effect on the elevation of DHA and suppression of 22:5 ω -6 by DHASCO.

The pattern of fatty acid changes in liver tissue (Fig. 5) were nearly identical to those in the heart tissue. In liver tissue, however, EPA levels were higher and found to be significantly reduced in the ARASCO-supplemented group. EPA was significantly increased in the DHASCO-supplemented group and unchanged if both oils were added together. Although the effect of ARASCO and DHASCO on EPA was opposite to that on ARA, the additive effect of the oils was similar in that the effects seemed to be cancelled out.

The fatty acid changes due to the various dietary supplements observed in the testes (Fig. 6) were similar to that in brain tissue. Although the only significant changes occurred in the 22:4 ω -6 composition, changes consistent with those in liver and heart occurred, but did not achieve statistical significance. This may be a result of a wider degree

of variability since only half the number of samples (only males) could be used for these analyses.

DISCUSSION

An acute toxicity test and a 4-wk oral gavage toxicity test were carried out in rats to evaluate the safety of the triglyceride oils ARASCO and DHASCO. The combination of these oils, Formulaid, was also tested in the 4-wk oral gavage study. On the basis of the results, the estimated median lethal dose for both ARASCO and DHASCO is greater than 20 g/kg body weight, the maximum dose physically possible in these animals. Assuming an average consumption of 5 g fat/kg/day for infants (Jensen, 1992), and a 1:100 dilution of ARASCO and a 1:200 dilution of DHASCO to achieve the ARA and DHA levels of human milk [0.4% and 0.2%, respectively (Jensen *et al.*, 1992)], then this represents an 800-fold safety margin. In the 4-wk oral gavage study, at doses of 2.5 g/kg/day, 1.25 g/kg/day and 3.75 g/kg/day for ARASCO, DHASCO and

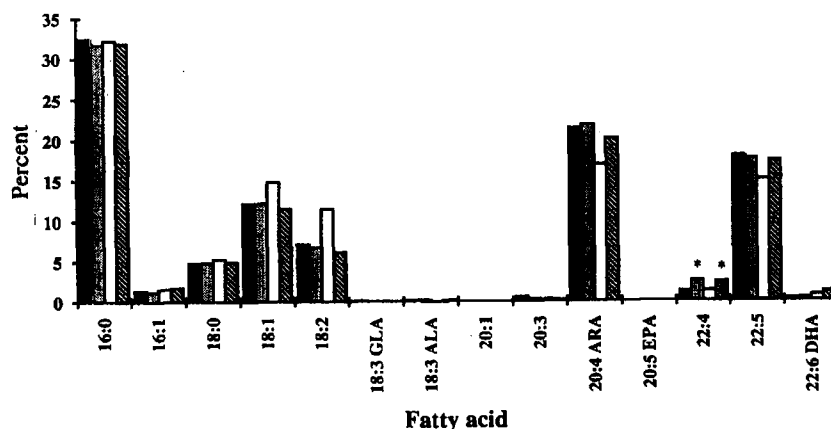


Fig. 6. Fatty acid composition of testes tissue from rats in the high-dose ARASCO (■), DHASCO (□) and Formulaid (▨) groups, and the control group (■). * $P \leq 0.05$ v. control values (two-tailed Student's *t*-test). ALA = α -linolenic acid; GLA = γ -linolenic acid.

Formulaid, respectively, all animals survived to the scheduled sacrifice, and no clinical signs due to administration of the test materials were evident. On the basis of the aforementioned calculations, this represents a 50-fold safety margin over an intended use level for infant formula. These results represent the first published safety evaluations of single-cell oils and may, in turn, offer evidence towards developing a new safe source of ARA and DHA as dietary supplements.

The tissue fatty acid analyses of the brain, heart, liver and testes show that ARA and DHA are bioavailable from ARASCO, DHASCO and Formulaid. As these animals were not fatty acid deficient or deprived at an early stage of development, there were no significant differences in ARA or DHA levels in the brain tissue of animals in the ARASCO, DHASCO and Formulaid groups compared with that in the control animals which were fed high oleic sunflower oil. There was, however, a significant increase in the 22:4 ω -6 content in the tissues of animals in the ARASCO and Formulaid groups. This was most likely caused by elongation of ARA (from ARASCO and Formulaid). There was also a significant decrease in fatty acid 18:2 in selected tissues of these two groups possibly caused by feedback inhibition of the desaturases and elongases involved in biosynthesis of ARA (Brenner, 1969).

The heart and liver fatty acid compositions responded more readily to test material administration. As expected, those animals that received ARASCO had significantly greater ARA levels in their heart and liver tissues, and those animals administered DHASCO had significantly greater levels of DHA in their heart and liver tissues. DHASCO administered alone also decreased the level of ARA and 22:4 ω -6 in the heart and liver tissues. However, when the animals were given Formulaid, a combination of ARASCO and DHASCO, DHA levels were significantly elevated without decreasing the levels of ARA. This is important for supplementation of infant formula with DHA because decreased ARA resulting from fish oil supplementation (DHA and EPA) led to growth retardation in premature infants (Carlson, 1991; Carlson *et al.*, 1992). In heart tissue, there were significant decreases in the levels of 18:1 and 18:2 in animals in the ARASCO and Formulaid groups which may also be a result of feedback inhibition. In liver tissue, there was also a significant decrease in the level of 20:5 ω -3 in the ARASCO group and significant decreases in the levels of 18:1, 18:2 in the ARASCO and Formulaid groups. The significant increase in 22:4 ω -6 in liver tissue of the ARASCO group was likely to be the result of elongation of ARA. The apparent decrease in 22:5 ω -6 in the heart and liver tissues of animals in the DHASCO and Formulaid groups is more likely explained by an elevation in the levels of 22:5 in the control and ARASCO groups as is typically found when animals are ω -3 deficient. (Galli *et al.*, 1971).

In man, the testes have a very high level of DHA. However, in rats, the testes contain high levels of 22:5 and low levels of DHA (Bieri *et al.*, 1969). Although feeding fish oil has been shown to reverse the levels of 22:5 and DHA in rats (Ayala *et al.*, 1977), feeding DHASCO and Formulaid only slightly increased the levels of DHA in the testes of rats. The only significant change in the testis tissue was an increase in 22:4 ω -6 in animals in the ARASCO and Formulaid supplementation groups. As before, the authors believe this to simply be caused by elongation of ARA. Although there appears to be a significant increase in the levels of 18:1 and 18:2 in the DHASCO group, the standard deviations were large, and the results were not statistically significant.

It is concluded from these studies that ARASCO, DHASCO and Formulaid have no demonstrable toxicity at the doses tested and that administration of these single-cell oils is no less safe than administration of sunflower oil. Furthermore, ARA and DHA in these oils are both highly bioavailable. As ARASCO and DHASCO are enriched in a single bioactive fatty acid, they may offer a new source of these fatty acids for nutritional purposes. In addition, the ability to elevate levels of DHA in tissues without decreasing the levels of ARA is a unique advantage of Formulaid. Together with the fact that it is a triglyceride oil, the authors believe that Formulaid will represent one important new component of infant formula.

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Highly purified eicosapentaenoic acid and docosahexaenoic acid in humans have similar triacylglycerol-lowering effects but divergent effects on serum fatty acids¹⁻³

Sameline Grimsgaard, Kaare H Bønaa, John-Bjarne Hansen, and Arne Nordøy

ABSTRACT To compare the effects of highly purified ethyl ester concentrates of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on serum lipids, apolipoproteins, and serum phospholipid fatty acids in humans, we conducted a double-blind, placebo-controlled, parallel design intervention study. Healthy nonsmoking men ($n = 234$) aged 36–56 y were randomly assigned to dietary supplementation with 3.8 g EPA/d, 3.6 g DHA/d, or 4.0 g corn oil/d (placebo) for 7 wk. Serum triacylglycerols decreased 26% ($P < 0.0001$) in the DHA group and 21% ($P = 0.0001$) in the EPA group compared with the corn oil group. Although not significant, net decreases in serum triacylglycerols were consistently greater in the DHA group across all quartiles of baseline triacylglycerol concentrations. Serum high-density-lipoprotein cholesterol increased 0.06 mmol/L ($P = 0.0002$) in the DHA group. In the EPA group, serum total cholesterol decreased 0.15 mmol/L ($P = 0.02$) and apolipoprotein A-I decreased 0.04 g/L ($P = 0.0003$). In the DHA group, serum phospholipid DHA increased by 69% and EPA increased by 29%, indicating retro-conversion of DHA to EPA. In the EPA group, serum phospholipid EPA increased by 297% whereas DHA decreased by 15%, suggesting that EPA is not elongated to DHA in humans. The serum phospholipid ratio of $n-3$ to $n-6$ fatty acids increased in both groups, whereas the relative changes in $n-6$ fatty acids suggested possible alterations in liver desaturation activity in the DHA group. We conclude that both DHA and EPA decrease serum triacylglycerols, but have differential effects on lipoprotein and fatty acid metabolism in humans. *Am J Clin Nutr* 1997;66:649–59.

KEY WORDS Fatty acids, $n-3$ fatty acids, eicosapentaenoic acid, docosahexaenoic acid, triacylglycerols, phospholipids, randomized controlled trials

INTRODUCTION

Accumulating evidence indicates that fish oil, rich in eicosapentaenoic acid (EPA; 20:5 $n-3$) and docosahexaenoic acid (DHA; 22:6 $n-3$) of the $n-3$ family, can modify a variety of cellular processes associated with lipid metabolism, atherosclerosis, hypertension, thrombosis, and inflammation (1). The amount and the ratio of DHA to EPA in different marine sources vary considerably (1, 2). Earlier studies of $n-3$ fatty acid supplementation in humans used

oils varying in dosage form, total dose of fatty acids, and relative content of DHA and EPA. Examination of these data shows that the most consistent effect of $n-3$ fatty acids on cardiovascular disease risk factors is a reduction in serum triacylglycerol concentration, whereas reported effects on other variables are less consistent (3–5). It is possible that the inconsistencies derive from chance findings in small-scale studies or differences in study design. However, they may also be attributed to varying metabolic effects of DHA and EPA.

Animal studies showed that EPA and DHA accumulate in different compartments in the body and thus may be subject to differences in both metabolism and effects (6–8). DHA selectively attenuated expression of proatherogenic and proinflammatory proteins in human endothelial cells, suggesting a beneficial effect of DHA on atherosclerosis (9), whereas EPA may be a more potent platelet inhibitor than DHA (10, 11). In vitro studies indicate that EPA and DHA have different effects on triacylglycerol synthesis (12), and it was suggested that EPA is primarily responsible for the hypotriacylglycerolemic effect of $n-3$ fatty acids both in rats (13) and humans (14). The extent to which these reports can be generalized is constrained by limitations in study design, however. Knowledge of the specific effects of EPA and DHA is needed to target $n-3$ supplements for specific effects. Long-term studies with adequate sample size comparing the biological effects of pure DHA and EPA in human volunteers have not been reported (10, 14–16). We therefore conducted a double-blind, randomized, placebo-controlled, parallel design intervention study to evaluate effects of dietary supplementation with highly purified EPA or DHA on serum lipids, apolipoproteins, and serum phospholipid fatty acid composition.

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SUBJECTS AND METHODS

Subjects and experimental design

In 1986–1987, 21 826 subjects, 81.3% of the men aged 20–61 y old and the women aged 20–56 y old living in the municipality of Tromsø, participated in a health survey (visit 1) (17). All subjects completed a questionnaire about previous disease, use of drugs, and diet and smoking habits, and their height, weight, blood pressure, and nonfasting serum lipid concentrations were measured. Four hundred seven men between the ages of 35 and 55 were selected according to the following criteria: they reported being healthy nonsmokers, did not use nonprescribed or prescribed drugs, and consumed less than four fish dishes per week in their usual diet. They also had serum cholesterol concentrations < 8.0 mmol/L, diastolic blood pressure < 95 mm Hg, and systolic blood pressure < 160 mm Hg. These men were then asked in 1993 to undergo a clinical examination that included a complete medical history, physical examination, and laboratory tests.

Among the 349 men who responded to the invitation, 251 subjects filled the above-mentioned criteria and were recruited into the present study. They had no cardiovascular, liver, or renal disease; bleeding disorder; diabetes mellitus; psychopathologic disease; alcoholism; or other disease that can influence blood pressure, lipid metabolism, or hemostasis. They were not consuming special diets and did not expect to change their diet or lifestyle during the study period. Their mean ($\pm$ SD) age was 44 ± 5 y (range: 36–56). The study was approved by the regional board of research ethics, and each subject gave informed consent.

The study was performed according to Good Clinical Practice requirements (18). It began with a 4-mo run-in period during which subjects were asked to continue their usual diet and living habits and during which their blood pressure and fasting serum lipid concentrations were measured on two occasions (visit 2 and visit 3). Each subject's average intake of nutrients was calculated on a fourth visit. At the beginning of the run-in period and throughout the study, participants were instructed not to ingest cod liver oil or other fish-oil supplements.

For entry into the intervention phase of the study, a subject's mean serum triacylglycerol concentration during the run-in period had to be < 5.0 mmol/L and mean serum cholesterol concentration < 9.5 mmol/L. Among the 251 subjects, 2 were smokers, 2 had serum glucose or triacylglycerol concentrations above the inclusion criteria, 2 used cardiovascular drugs, 1 consumed more than three fish dishes per week, and 10 dropped out during the run-in period for personal reasons. Thus, 234 men entered the double-blind, parallel group intervention trial, which lasted for 7 wk. Computer-generated random numbers were used to assign the participants to either 4.0 g 95% ethyl ester EPA/d, 4.0 g 90% ethyl ester DHA/d, or 4.0 g corn oil/d. The dietary supplements were administered in indistinguishable soft gelatin capsules that each contained 1.0 g oil and 4–6 IU vitamin E as an antioxidant (Table 1). Each individual was asked to ingest two capsules in the morning and two capsules at night. The dietary supplements were manufactured by Pronova Biocare AS, Oslo.

Participants were examined after an overnight fast between 0800 and 1130 on two separate occasions separated by an

TABLE 1

Composition of dietary supplements¹

Constituent	DHA	EPA	Corn oil
22:6n-3 Ethyl ester (mg)	889	12	0
20:5n-3 Ethyl ester (mg)	18	941	0
18:2n-6 (mg)	0	0	559
18:1n-9 (mg)	0	0	259
Vitamin E (IU)	4–6	4–6	3.7
p-Anisidine value	<35	<35	—
Peroxide value (mmol/g)	<0.01	<0.01	—

¹ Dietary supplements were given in indistinguishable, oblong, soft gelatin capsules of 1.4 g average weight. DHA, docosahexaenoic acid supplement; EPA, eicosapentaenoic acid supplement.

interval of 3–5 d, both at baseline (visits 5 and 6) and after 7 wk of supplementation (visits 7 and 8). At each visit blood pressure was measured and blood samples were collected. Participants were asked to abstain from alcohol and strenuous exercise for 48 h before the visit. A telephone interview was performed in the middle of the intervention period to monitor study compliance, side effects, and intercurrent disease. Compliance was assessed by counting leftover capsules and was calculated as the percentage of the prescribed capsules taken. We also measured serum phospholipid fatty acid concentrations at baseline and at the end of intervention.

Clinical and laboratory measurements

Height was measured during the run-in period and weight was measured at baseline and after the intervention period on an electronic scale with subjects wearing light clothing and no shoes. Before the intervention each subject's habitual nutrient intake was assessed during a 1-h interview by a certified clinical nutritionist using the dietary history method. Food models and containers were used to estimate quantities. Dietary constituents were calculated from standard food tables that also cover individual fatty acids by using a specially designed computer program (19–22). Each subject completed a self-administered questionnaire at baseline and during the last week of the intervention to monitor food habits and physical activity during the intervention. Participants were asked how many times they ate fish or meat for dinner and how many units of alcohol they consumed during the past week (one unit of alcohol equals 9 g). Those who reported being physically active four or more times weekly for ≥ 20 min, leading to sweating or shortness of breath, were categorized as active; those reporting 1–3 times weekly were categorized as moderately active; and those reporting 0 times weekly were categorized as sedentary.

Blood samples were drawn from an antecubital vein into an evacuated tube system using minimal stasis. Serum was prepared by clotting whole blood in a glass tube (Becton Dickinson, Meylan Cedex, France) at room temperature for 1 h and then centrifuging the sample at $2000 \times g$ for 15 min at 22 °C. One-milliliter aliquots of serum were transferred into sterile 2-mL cryovials (Corning, Park Ridge, IL), flushed with nitrogen, and stored at -70 °C. Blood for plasma preparation was collected into vacutainers (Becton Dickinson) containing 0.129 mol sodium citrate/L (blood:anticoagulant = 10:1). Plasma was prepared by centrifugation at $2000 \times g$ for 15 min at 22 °C, transferred into sterile cryovials in aliquots of 1 mL,



flushed with nitrogen, and stored at -70°C . All blood samples were analyzed after completion of the intervention period and before the randomization code was broken.

Serum lipids were analyzed on a Hitachi 737 Automatic Analyzer (Boehringer Mannheim, Mannheim, Germany) with reagents from the manufacturer. Total cholesterol was measured with an enzymatic colorimetric method (CHOD-PAP) and high-density-lipoprotein (HDL) cholesterol was assayed by the same procedure after precipitation of lower-density lipoproteins with heparin and manganese chloride. Serum triacylglycerol concentrations were determined with an enzymatic colorimetric test (GPO-PAP). Low-density-lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula (23). Apolipoprotein A-I and apolipoprotein B-I were measured immunochemically by rate nephelometry using the Array Protein System from Beckman Instruments Inc (Brea, CA).

Fatty acids were measured by extracting total lipids from 500 μL serum according to Folch et al (24), with phosphatidylcholine diheptadecanoyl added as an internal standard (P-5014; Sigma Chemical Company, St Louis), chloroform:methanol (2:1, by vol) as a solvent, and butylated hydroxytoluene (75 mg/L) as an antioxidant. Total phospholipids were separated by solid-phase extraction with NH_2 columns (size 3 cc; Analytische Bond Elut LRC; Varian, Harbour City, CA) (25), followed by transmethylation with boron trifluoride, extraction into hexane, and evaporation to dryness. The fatty acid methyl esters were dissolved in hexane and analyzed by gas-liquid chromatography (Shimadzu GC-14 A; Shimadzu Corporation, Kyoto, Japan) fitted with a capillary column (CP-Sil 88; length: 50 m, internal diameter: 0.25 mm) obtained from Chrompack Inc (Raritan, NJ). Retention times and response factors for each fatty acid were determined using standards obtained from Nu-Chek Prep (Elysian, MN). The results were integrated on a Shimadzu C-R4A integrator. Fatty acid concentrations are reported as μmol fatty acid/L serum.

Statistical analysis

All results are expressed as means $\pm$ SDs. On examination of the frequency distributions, all variables except serum triacylglycerol and certain lifestyle factors such as level of physical activity and fish, meat, and alcohol consumption were normally distributed at baseline and at the end of intervention. Serum lipid concentrations at baseline and at the end of the intervention were calculated as the mean of the values obtained at visits 5 and 6 and the mean of the values obtained at visits 7 and 8, respectively. Change was calculated as the value obtained after intervention minus the value obtained at baseline. Percentage change was calculated as the group-wise mean percentage change from baseline. Because of missing values, change could not be calculated for some individuals. Analysis of changes in serum lipids, serum phospholipid 16:1n-7, and sum of serum phospholipid fatty acids are therefore based on 222, 217, and 209 subjects, respectively. Two influencing outlying values were excluded from the analysis of desaturation indexes.

To evaluate within-group change, we used paired *t* tests for normally distributed variables, the Wilcoxon signed-rank test for ordinal and non-normally distributed variables, and the chi-square statistic for categorical variables. One-way analysis of variance was used to evaluate whether change differed between groups; the *F* test was used for normally distributed

variables and the Kruskal-Wallis test for ordinal and non-normally distributed variables. Between-group comparisons of change were done by contrasting groups in the SAS general linear model procedure when the overall *F* test was significant at $P < 0.05$ (26). We did not adjust for multiple comparisons (27). Results were considered significant when the two-sided *P* value was < 0.05 . Caution should be applied when interpreting *P* values in the present study because three contrasts were tested. When applying the Tukey multiple-comparison procedure (28), the 95% CI included the null value of no effect for those contrasts for which the unadjusted *P* value was > 0.03 . Correlations were tested by computing Pearson or Spearman correlation coefficients.

RESULTS

Three of the 234 subjects who were randomly assigned to a study arm dropped out during the intervention period. One subject in the DHA group was found to have fat intolerance after cholecystectomy, one subject in the EPA group developed diarrhea, and one subject in the corn oil group experienced vertigo and vomiting that was considered unrelated to the dietary supplements. Two individuals in the DHA group, three in the EPA group, and two in the corn oil group were excluded from the analysis. The reasons for exclusions were possible renal disease ($n = 1$), poor compliance with study protocol ($n = 1$), initiation of a vasoactive drug ($n = 1$), cancer surgery ($n = 1$), and change in amount of physical activity during the intervention ($n = 3$). Thus, 224 subjects are included in the present analysis. Mean ages of the subjects were 43 ± 5 , 44 ± 5 , and 45 ± 6 y and mean body mass indexes (in kg/m^2) were 24.9 ± 2.6 , 25.6 ± 2.9 , and 24.6 ± 2.7 in the DHA, EPA, and corn oil groups, respectively.

There were no significant changes in hematology, blood chemistry (electrolytes, alanine aminotransferase, γ -glutamyl transferase, alkaline phosphatase, albumin, bilirubin, creatinine, and C-reactive protein), serum glucose, or plasma-active renin after dietary intervention with DHA, EPA, or corn oil (data not shown).

Compliance and side effects

The mean number of days in the study was 49 ± 5 , 48 ± 3 , and 48 ± 4 d in the DHA, EPA, and corn oil groups, respectively. Percentage compliance was slightly poorer in the DHA group ($91 \pm 6\%$) compared with the EPA and corn oil groups (both $94 \pm 6\%$). There were no within-group correlations between compliance and change in serum DHA, EPA, or linoleic acid concentrations.

Side effects were mild and transient and for most individuals faded 1–2 wk after the start of the intervention. Fifty-eight percent of subjects in the DHA group and 57% in the EPA group experienced belching after initiation of the dietary supplements compared with 4% in the corn oil group. A taste of fish oil during the intervention was reported by 67% of subjects in the DHA group, 65% in the EPA group, and 3% in the corn oil group.

Diet, body weight, and physical activity

The DHA, EPA, and corn oil groups were well balanced at baseline. Total fat accounted for 30% of energy intake in all



TABLE 2

Composition of background diet¹

Daily nutrient intake	DHA (n = 72)	EPA (n = 75)	Corn oil (n = 77)
Energy (kJ)	10 370 ± 2561	10 223 ± 2170	10 877 ± 2455
Protein (g)	103 ± 23	103 ± 22	107 ± 25
Carbohydrate (g)	335 ± 80	324 ± 74	349 ± 87
Fiber (g)	24.1 ± 7.3	23.2 ± 6.7	25.2 ± 7.7
Alcohol (g)	5.82 ± 6.28	6.39 ± 6.56	7.04 ± 7.00
Total fat (g)	81.2 ± 32.1	81.6 ± 23.9	85.9 ± 27.0
Cholesterol (mg)	314 ± 102	327 ± 89	334 ± 101
Saturated fat (g)	33.6 ± 12.4	34.3 ± 10.9	35.4 ± 11.4
Monounsaturated fat (g)	27.6 ± 11.3	28.0 ± 8.7	29.6 ± 9.6
Polyunsaturated fat (g)	13.5 ± 8.4	12.7 ± 4.6	14.0 ± 6.1
P:S	0.40 ± 0.15	0.39 ± 0.13	0.40 ± 0.13
18:2n-6 (g)	10.2 ± 7.0	9.50 ± 3.70	10.7 ± 5.1
20:5n-3 (g)	0.18 ± 0.20	0.19 ± 0.18	0.19 ± 0.21
22:6n-3 (g)	0.34 ± 0.32	0.35 ± 0.28	0.36 ± 0.32
β-Carotene (μg)	2651 ± 1902	2634 ± 1284	2749 ± 1905
Retinol (μg)	1003 ± 955	993 ± 717	1031 ± 697
Thiamine (mg)	1.66 ± 0.38	1.67 ± 0.35	1.76 ± 0.43
Riboflavin (mg)	2.20 ± 0.70	2.29 ± 0.67	2.31 ± 0.72
Niacin (mg)	23.6 ± 4.8	23.2 ± 4.8	23.8 ± 5.2
Vitamin C (mg)	90.0 ± 1.6	78.6 ± 35.5	88.9 ± 45.0
Vitamin D (μg)	6.08 ± 9.77	5.33 ± 4.16	5.65 ± 3.81
Vitamin E (mg)	4.87 ± 1.48	4.96 ± 1.34	5.33 ± 1.53

¹ $\bar{x} \pm$ SD. DHA, docosahexaenoic acid group; EPA, eicosapentaenoic acid group; P:S, ratio of polyunsaturated to saturated fatty acids.

groups. Dietary intake of DHA and EPA at baseline accounted for 0.7% of total fat intake. Differences in nutrient intake between the DHA, EPA, and corn oil groups were minor and not significant (Table 2). No significantly different within- or between-group changes were found with respect to body weight, physical activity, or food habits during the intervention (Table 3). Body weight increased by 0.6 kg in the corn oil group and by 0.7 kg in the DHA and EPA groups. There was a nonsignificant increase in the percentage of participants who reported being sedentary after compared with before the intervention. Alcohol, meat, and fish consumption (dinner meals) increased slightly but not significantly during the intervention. None of the participants reported consuming more than three

fish dishes weekly before or during the intervention. There was good agreement between measures of alcohol consumption obtained by the nutritionist during the run-in period and by the self-administered questionnaire at baseline ($r = 0.73$, $P = 0.0001$).

Serum lipids and apolipoproteins

Serum mean (95% CI in parentheses) triacylglycerol concentrations decreased 0.22 mmol/L (0.15, 0.29) in the DHA group and 0.15 mmol/L (0.06, 0.24) in the EPA group (Table 4). In the corn oil group serum triacylglycerols increased 0.11 mmol/L (0.03, 0.19). Compared with change for the corn oil group, serum triacylglycerols decreased 26% in the DHA group and 21% in the EPA group. The difference between the DHA and EPA groups was not significant ($P = 0.14$). However, net decreases in serum triacylglycerols were consistently greater in the DHA group than in the EPA group across quartiles of baseline triacylglycerol concentrations (Table 5). In the EPA and DHA groups there were no correlations between changes in individual n-3 fatty acids and changes in serum triacylglycerol concentrations.

Serum total cholesterol decreased 0.15 mmol/L ($P < 0.05$) in the EPA group and apolipoprotein A-I decreased 0.04 g/L ($P < 0.001$, Table 4). These changes differed significantly from both the DHA and the corn oil groups. In the DHA group, HDL cholesterol increased 0.06 mmol/L ($P < 0.001$), differing significantly from both the EPA and corn oil groups. Hence, in both the EPA and DHA groups there was an increase in the ratio of HDL cholesterol to apolipoprotein A-I and a decrease in the ratio of total cholesterol to HDL cholesterol.

Serum phospholipid fatty acid concentrations

In the total study group ($n = 224$), the correlations between dietary intake and serum phospholipid concentrations of DHA and EPA at baseline were $r = 0.39$ and $r = 0.35$, respectively (both $P = 0.0001$). The mean of individual ratios of dietary DHA to EPA at baseline was 2.5 ± 1.2 , whereas the serum phospholipid ratio of DHA to EPA was 3.8 ± 1.6 ($P = 0.0001$, for the difference between the ratios), indicating accumulation of DHA relative to EPA in serum phospholipids.

TABLE 3

Body weight and lifestyle factors at baseline and change after 7 wk of supplementation with docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), or corn oil¹

	DHA (n = 72)		EPA (n = 75)		Corn oil (n = 77)	
	Baseline	Change	Baseline	Change	Baseline	Change
Body weight (kg)	80.0 ± 10.0 ²	0.7 ± 1.2	82.6 ± 10.0	0.7 ± 1.4	79.5 ± 9.4	0.6 ± 1.1
Fish consumption (dishes/wk)	2.10 ± 1.01	0.06 ± 1.13	2.16 ± 1.05	0.16 ± 1.06	2.03 ± 1.10	0.21 ± 1.29
Meat consumption (dishes/wk)	2.46 ± 1.31	0.24 ± 1.53	2.56 ± 1.39	0.16 ± 1.23	2.93 ± 1.28	0.15 ± 2.01
Teetotalers (%)	4	0	1	0	4	0
Alcohol consumption (g/wk) ³	45.3 ± 44.3	0.3 ± 5.1	59.6 ± 63.9	-0.8 ± 6.5	55.5 ± 50.8	1.5 ± 7.0
Physical activity						
Sedentary (%)	22	3	26	1	25	6
Moderate (%)	69	-6	59	-1	54	1
Active (%)	9	3	15	0	21	-7

¹ There were no significant differences among groups.

² $\bar{x} \pm$ SD.

³ Teetotalers were excluded from analysis of alcohol consumption.

TABLE 4

Serum lipids and apolipoproteins at baseline and change after 7 wk of supplementation with docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), or corn oil

	DHA (n = 72)		EPA (n = 75)		Corn oil (n = 77)		F test: P ¹	Contrasts between groups: P		
	Baseline	Change	Baseline	Change	Baseline	Change		DHA vs EPA	DHA vs corn oil	EPA vs corn oil
Triacylglycerols (mmol/L)	1.24 ± 0.58 ²	-0.22 ± 0.31 ³	1.23 ± 0.57	-0.15 ± 0.40 ⁴	1.22 ± 0.55	0.11 ± 0.34 ⁴	0.0001	0.14	0.0001	0.0001
Total cholesterol (mmol/L)	6.00 ± 0.95	0.03 ± 0.49	5.98 ± 0.94	-0.15 ± 0.55 ⁵	6.02 ± 1.08	0.10 ± 0.55	0.01	0.04	0.4	0.004
LDL cholesterol (mmol/L)	4.06 ± 0.86	0.07 ± 0.46	4.06 ± 0.83	-0.08 ± 0.48	4.04 ± 0.98	0.06 ± 0.48	0.10	—	—	—
HDL cholesterol (mmol/L)	1.36 ± 0.30	0.06 ± 0.13 ³	1.33 ± 0.31	0.01 ± 0.12	1.41 ± 0.28	-0.01 ± 0.11	0.001	0.009	0.0005	0.4
Apolipoprotein A-I (g/L)	1.38 ± 0.21	0.02 ± 0.13	1.38 ± 0.20	-0.04 ± 0.10 ³	1.46 ± 0.23	0.00 ± 0.12	0.003	0.0008	0.3	0.02
Apolipoprotein B (g/L)	1.00 ± 0.21	-0.01 ± 0.11	1.01 ± 0.23	-0.03 ± 0.11 ⁵	1.02 ± 0.28	0.02 ± 0.11	0.05	—	—	—
HDL:apolipoprotein A-I	0.97 ± 0.14	0.04 ± 0.07 ³	0.96 ± 0.13	0.04 ± 0.08 ³	0.97 ± 0.12	-0.01 ± 0.06	0.0001	0.8	0.0003	0.0001
Total:HDL cholesterol	4.62 ± 1.19	-0.19 ± 0.52 ⁴	4.70 ± 1.24	-0.13 ± 0.47 ⁵	4.43 ± 1.19	0.11 ± 0.62	0.002	0.4	0.0006	0.007

¹ ANOVA for between-group comparisons of change.

² $\bar{x}$ ± SD.

³⁻⁵ One-sample t test of difference between baseline and 7 wk: ³ P < 0.001, ⁴ P < 0.01, ⁵ P < 0.05.

TABLE 5

Change in serum triacylglycerol concentration in docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and corn oil groups according to quartiles of baseline triacylglycerol concentration

Baseline triacylglycerol concentration	Change in serum triacylglycerol concentration			Estimated n-3 fatty acid effect ¹	
	DHA (n = 72)	EPA (n = 75)	Corn oil (n = 77)	DHA	EPA
	mmol/L			mmol/L (%)	
1st quartile:					
0.69 (0.34-0.82) mmol/L ²	0.00 ± 0.13 ³	0.03 ± 0.21	0.10 ± 0.21	-0.10 (-14)	-0.07 (-10)
2nd quartile:					
0.96 (0.83-1.09) mmol/L	-0.14 ± 0.18	-0.04 ± 0.26	0.15 ± 0.28	-0.29 (-30)	-0.19 (-20)
3rd quartile:					
1.24 (1.10-1.44) mmol/L	-0.16 ± 0.23	-0.03 ± 0.34	0.14 ± 0.32	-0.30 (-24)	-0.17 (-14)
4th quartile:					
2.01 (1.45-3.61) mmol/L	-0.56 ± 0.35	-0.52 ± 0.46	0.03 ± 0.50	-0.59 (-29)	-0.55 (-27)

¹ Effect attributable to DHA and EPA; ie, change in DHA group minus change in corn oil group and change in EPA group minus change in corn oil group.

² $\bar{x}$; range in parentheses.

³ $\bar{x}$ ± SD.

The total amount of serum phospholipid fatty acids did not change between groups during EPA, DHA, or corn oil supplementation (Table 6). Likewise, there were no changes in serum phospholipid saturated fatty acids. As for the monounsaturated fatty acids, palmitoleic acid (16:1n-7) decreased significantly by 20% in the DHA group, compared with no change in the EPA or corn oil groups. Oleic acid (18:1n-9) concentrations decreased by 11% and 12% in the DHA and EPA groups, respectively.

The total serum phospholipid n-6 fatty acid concentration (sum of 18:2n-6, 20:3n-6, and 20:4n-6) decreased more in the EPA (-23%) than in the DHA (-11%) group. In the DHA group, however, the ratio between the individual n-6 fatty acids changed more than in the EPA group. The ratio of 20:4n-6 + 20:3n-6 to 18:2n-6 can be used as an index of $\Delta 6$ desaturation activity because changes in $\Delta 5$ desaturation will not influence the ratio. The $\Delta 6$ desaturation index decreased significantly in the DHA group compared with no change in the EPA or corn oil group (Table 7). Similarly, the ratio of 20:4n-6 to 20:3n-6 + 18:2n-6 can be used as an index of $\Delta 5$ desaturation activity. This ratio decreased significantly in the DHA group whereas it increased significantly in the EPA group. As a result, the ratio of arachidonic (20:4n-6) to linoleic (18:2n-6) acid decreased in the DHA group and increased in the EPA group.

During the intervention, the serum phospholipid concentration of n-3 fatty acids increased by 47% in the DHA group and by 68% in the EPA group. The concentration of α -linolenic acid (18:3n-3) decreased in both the DHA and EPA groups compared with the corn oil group (Table 6). In the DHA group, mean serum phospholipid DHA and EPA concentrations increased significantly by 69% (individual range: -33% to 669%) and 29% (individual range: -63% to 557%), respectively, whereas docosapentaenoic acid (DPA; 22:5n-3) decreased by 33% (individual range: -72% to 221%). In this group, the correlation between the change in serum DHA and EPA was $r = 0.30$ ($P = 0.01$, Figure 1). In the EPA group, serum phospholipid EPA increased by 297% (individual range: -2% to 1196%) and docosapentaenoic acid by 130% (individual range: -9% to 393%). Surprisingly, the serum phospholipid concentration of DHA decreased by 15% (individual range: -65% to 85%; $P < 0.001$) after EPA supplementation. The correlation between the change in serum DHA and EPA was $r = 0.39$ ($P = 0.0005$) during supplementation with EPA (Figure 2).

DISCUSSION

Numerous studies have examined the effects of marine n-3 fatty acids on lipid metabolism, but the separate effects of the two major n-3 fatty acids have remained largely unknown. The present report extends previous data by showing that both DHA and EPA lower serum triacylglycerol concentrations. DHA may be responsible for the increase in HDL cholesterol observed with some n-3 fatty acid supplements whereas EPA may produce a small decrease in serum total cholesterol. Our data further show that DHA and EPA produce different effects on the fatty acid composition of serum phospholipids. We studied a fairly large sample

recruited from the general population and compliance with the study protocol was good. The generalizability of the study therefore appears sound.

It is well established that n-3 fatty acids lower serum triacylglycerols, but this is the first study in humans showing that this effect is attributable to both EPA and DHA. Surprisingly, DHA consistently had a more pronounced triacylglycerol-lowering effect than EPA across all baseline concentrations of triacylglycerol (Table 5). These observations provide strong evidence that DHA has a triacylglycerol-lowering effect of its own and is not acting solely after retroconversion to EPA, because if that were the case, DHA would not be more potent than EPA. This finding contrasts with previous studies in rats, in which dietary supplementation of highly purified EPA lowered serum triacylglycerols whereas DHA had a modest effect if any (13, 29-31). The opposing findings may be dose related: the DHA and EPA supplements in the rat studies calculated as $\text{mg} \cdot \text{d}^{-1} \cdot \text{kg body wt}^{-1}$ are 10- to 30-fold larger than in the present study in humans (13, 29). The opposing findings may also depend on species differences because rats and humans differ with respect to lipid metabolism (32). Our finding is further opposed by results from a single-blind crossover study concluding that EPA is responsible for the triacylglycerol-lowering effect in humans (14). However, the study was small with only nine individuals in the DHA group, and the wash-out period was 2 wk, which is too short in dietary intervention trials with n-3 fatty acids (33).

Previous studies suggested that serum HDL cholesterol is better maintained with oil rich in DHA than oil rich in EPA (2, 34). The present data confirm these findings. The mechanism by which DHA increases HDL is not known. Serum triacylglycerols and HDL cholesterol were inversely correlated at baseline ($r = -0.42$, $P = 0.0001$), possibly due to plasma lipid transfer protein activity (35, 36). Interestingly, there was no correlation between changes in triacylglycerols and changes in HDL concentrations during supplementation with DHA ($r = -0.04$, $P = 0.73$), suggesting that DHA affects triacylglycerol and HDL metabolism through separate mechanisms. Much evidence indicates that n-3 fatty acids decrease serum triacylglycerols mainly by reducing hepatic very-low-density-lipoprotein (VLDL) synthesis and secretion (37, 38), but n-3 fatty acids may also increase the catabolic rate of VLDL (39). Moreover, it has been reported that lipid transfer protein activity decreased after n-3 fatty supplementation in humans (40), resulting in higher HDL-cholesterol concentrations and more triacylglycerols remaining in the VLDL fraction. Whether such effects can explain the increase in HDL cholesterol and the more pronounced triacylglycerol-lowering effect after DHA supplementation in the present study remains unknown.

In both groups receiving active treatment there was an increase in the ratio of HDL to apolipoprotein A-I. The finding suggests an increased surface to core ratio of the HDL molecule and a redistribution of the HDL subclasses toward the larger and more favorable HDL₂ as previously reported (34, 40, 41). Apparently, both n-3 fatty acids produce more cholesterol-rich HDL particles, DHA by increasing the HDL-cholesterol concentration and EPA by decreasing the apolipoprotein A-I concentration. In these healthy men with moderately high serum cholesterol concentrations, no convincing effects on total cholesterol were seen in either treatment group, although a 3%



TABLE 6

Serum phospholipid fatty acid concentration at baseline and change after 7 wk of supplementation with docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), or corn oil

Fatty acid	DHA (n = 72)		EPA (n = 75)		Corn oil (n = 77)		F test: <i>P</i> ¹	Contrasts between groups: <i>P</i>		
	Baseline	Change	Baseline	Change	Baseline	Change		DHA vs EPA	DHA vs corn oil	EPA vs corn oil
	$\mu\text{mol/L}$		$\mu\text{mol/L}$		$\mu\text{mol/L}$					
16:0	1450 $\pm$ 223 ²	-8 $\pm$ 168	1448 $\pm$ 221	-32 $\pm$ 174	1502 $\pm$ 290	16 $\pm$ 211	0.3	—	—	—
18:0	531 $\pm$ 113	-4 $\pm$ 77	524 $\pm$ 96	4 $\pm$ 77	543 $\pm$ 95	11 $\pm$ 83	0.5	—	—	—
16:1n-7	23.6 $\pm$ 10.9	-4.8 $\pm$ 11.7 ³	23.9 $\pm$ 18.0	-1.1 $\pm$ 13.1	28.1 $\pm$ 40.1	-4.8 $\pm$ 31.1	0.5	—	—	—
18:1n-9	344 $\pm$ 88	-37 $\pm$ 95 ⁴	340 $\pm$ 88	-41 $\pm$ 74 ³	348 $\pm$ 97	6 $\pm$ 90	0.001	0.8	0.003	0.001
22:1n-9	2.03 $\pm$ 3.31	-0.42 $\pm$ 3.41	2.34 $\pm$ 3.31	0.03 $\pm$ 2.51	1.79 $\pm$ 2.86	-0.13 $\pm$ 3.15	0.7	—	—	—
24:1n-9	74.5 $\pm$ 27.1	-0.5 $\pm$ 16.0	72.3 $\pm$ 24.9	-1.9 $\pm$ 18.5	75.9 $\pm$ 27.0	0.42 $\pm$ 24.76	0.8	—	—	—
18:2n-6	845 $\pm$ 185	-74 $\pm$ 184 ⁴	867 $\pm$ 168	-199 $\pm$ 128 ³	902 $\pm$ 160	21 $\pm$ 159	0.0001	0.0001	0.003	0.0001
20:3n-6	102 $\pm$ 32	-24 $\pm$ 26 ³	105 $\pm$ 29	-32 $\pm$ 23 ³	104 $\pm$ 32	7 $\pm$ 28 ³	0.0001	0.046	0.0001	0.0001
20:4n-6	253 $\pm$ 73	-39 $\pm$ 49 ³	260 $\pm$ 71	-48 $\pm$ 46 ³	261 $\pm$ 65	8 $\pm$ 52	0.0001	0.3	0.0001	0.0001
18:3n-3	7.72 $\pm$ 3.78	-1.13 $\pm$ 4.23 ⁵	8.30 $\pm$ 4.69	-2.07 $\pm$ 5.13 ³	7.66 $\pm$ 3.71	0.32 $\pm$ 4.00	0.005	0.2	0.05	0.001
20:5n-3	59.8 $\pm$ 53.7	17.6 $\pm$ 52.9 ⁴	61.4 $\pm$ 41.0	182.1 $\pm$ 91.1 ³	64.0 $\pm$ 40.3	-2.0 $\pm$ 40.7	0.0001	0.0001	0.07	0.0001
22:5n-3	32.0 $\pm$ 11.8	-10.6 $\pm$ 9.9 ³	33.9 $\pm$ 10.8	44.2 $\pm$ 25.8 ³	36.2 $\pm$ 10.2	1.6 $\pm$ 10.3	0.0001	0.0001	0.0001	0.0001
22:6n-3	185 $\pm$ 88	128 $\pm$ 87 ³	184 $\pm$ 65	-28 $\pm$ 48 ³	203 $\pm$ 69	-2 $\pm$ 52	0.0001	0.0001	0.0001	0.01
Sum of fatty acids ⁶	4117 $\pm$ 653	-100 $\pm$ 481	4102 $\pm$ 600	-142 $\pm$ 485 ⁵	4326 $\pm$ 696	55 $\pm$ 594	0.06	—	—	—
n-3:n-6 ⁷	0.24 $\pm$ 0.12	0.16 $\pm$ 0.16 ³	0.24 $\pm$ 0.10	0.28 $\pm$ 0.17 ³	0.25 $\pm$ 0.09	-0.01 $\pm$ 0.07	0.0001	0.0001	0.0001	0.0001

¹ ANOVA for between-group comparisons of change. ² $\bar{x} \pm \text{SD}$. ³⁻⁵ One-sample *t* test of difference between baseline and 7 wk: ³ *P* < 0.001, ⁴ *P* < 0.01, ⁵ *P* < 0.05. ⁶ Sum of fatty acids also includes 20:0, 22:0, 24:0, 20:1, 20:2, and 22:4n-6. ⁷ n-3:n-6 = [(18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3)/(18:2n-6 + 20:3n-6 + 20:4n-6)].

TABLE 7

Desaturation indexes at baseline and change after 7 wk of supplementation with docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), or corn oil

Index	DHA (n = 71)		EPA (n = 75)		Corn oil (n = 77)		F test: <i>P</i> ¹	Contrasts between groups: <i>P</i>		
	Baseline	Change	Baseline	Change	Baseline	Change		DHA vs EPA	DHA vs corn oil	EPA vs corn oil
$\Delta 9$ Desaturation index										
18:1n-9 to 18:0	0.65 $\pm$ 0.12 ²	-0.07 $\pm$ 0.14 ³	0.66 $\pm$ 0.15	-0.08 $\pm$ 0.11 ³	0.64 $\pm$ 0.12	0.01 $\pm$ 0.12	0.0001	0.8	0.0001	0.0001
16:1n-9 to 16:0	0.016 $\pm$ 0.006	-0.003 $\pm$ 0.007 ³	0.016 $\pm$ 0.013	0.000 $\pm$ 0.01	0.016 $\pm$ 0.018	-0.001 $\pm$ 0.010	0.1	—	—	—
$\Delta 6$ Desaturation index ⁴	0.42 $\pm$ 0.10	-0.04 $\pm$ 0.09 ³	0.43 $\pm$ 0.12	0.00 $\pm$ 0.10	0.41 $\pm$ 0.10	0.01 $\pm$ 0.06	0.0006	0.0009	0.0007	0.9
$\Delta 5$ Desaturation index ⁵	0.27 $\pm$ 0.07	-0.02 $\pm$ 0.06 ⁶	0.27 $\pm$ 0.07	0.02 $\pm$ 0.06 ⁷	0.26 $\pm$ 0.07	0.00 $\pm$ 0.04	0.0003	0.0001	0.08	0.02
Arachidonic:linoleic acid	0.30 $\pm$ 0.08	-0.02 $\pm$ 0.07 ⁷	0.31 $\pm$ 0.09	0.02 $\pm$ 0.07 ⁶	0.29 $\pm$ 0.08	0.00 $\pm$ 0.05	0.0007	0.0001	0.03	0.09

¹ ANOVA for between-group comparisons of change. ² $\bar{x} \pm \text{SD}$. ³⁻⁷ One-sample *t* test of difference between baseline and 7 wk: ³ *P* < 0.001, ⁶ *P* < 0.05, ⁷ *P* < 0.01. ⁴ $\Delta 6$ Desaturation index = [(20:4n-6 + 20:3n-6)/18:2n-6]. ⁵ $\Delta 5$ Desaturation index = [20:4n-6/(20:3n-6 + 18:2n-6)].

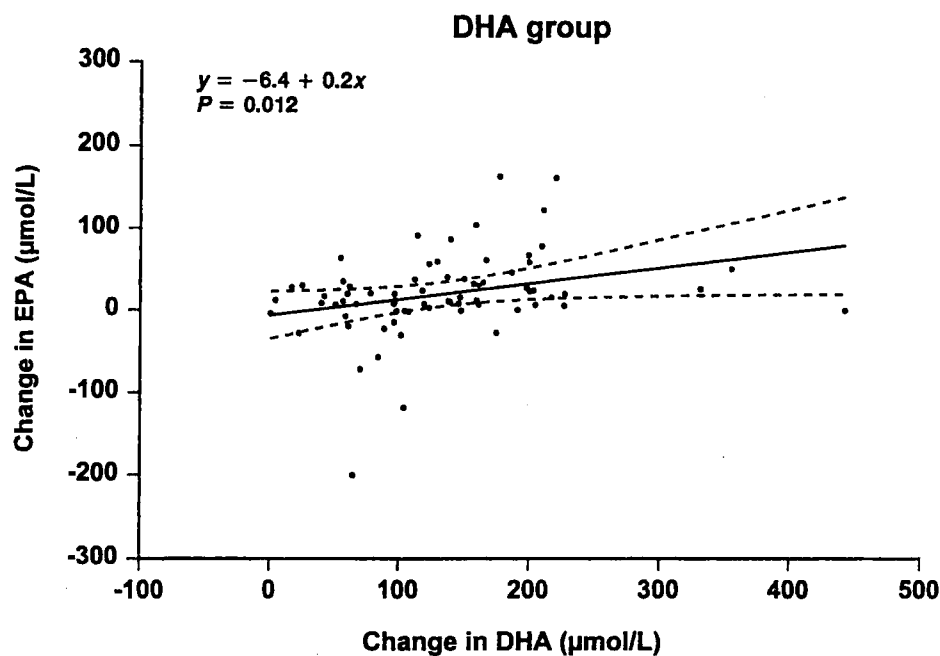


FIGURE 1. Plot of change in serum phospholipid docosahexaenoic acid (DHA) concentration versus change in serum phospholipid eicosapentaenoic acid (EPA) concentration in the DHA supplementation group ($n = 72$).

decrease was observed in the EPA group. This is in agreement with results obtained in previous studies concluding that $n-3$ fatty acids in moderate doses do not influence total cholesterol concentrations (4). There was, however, a beneficial decrease in the ratio of total cholesterol to HDL cholesterol in both the EPA and DHA groups.

Supplements with EPA or DHA produced an increase of serum phospholipid $n-3$ fatty acids at the expense of $n-6$ and monounsaturated fatty acids. In the EPA group, the percentage increase in serum phospholipid EPA (297%) was much larger than the corresponding increase in serum phospholipid DHA (69%) in the DHA group. A previous study showed that marine

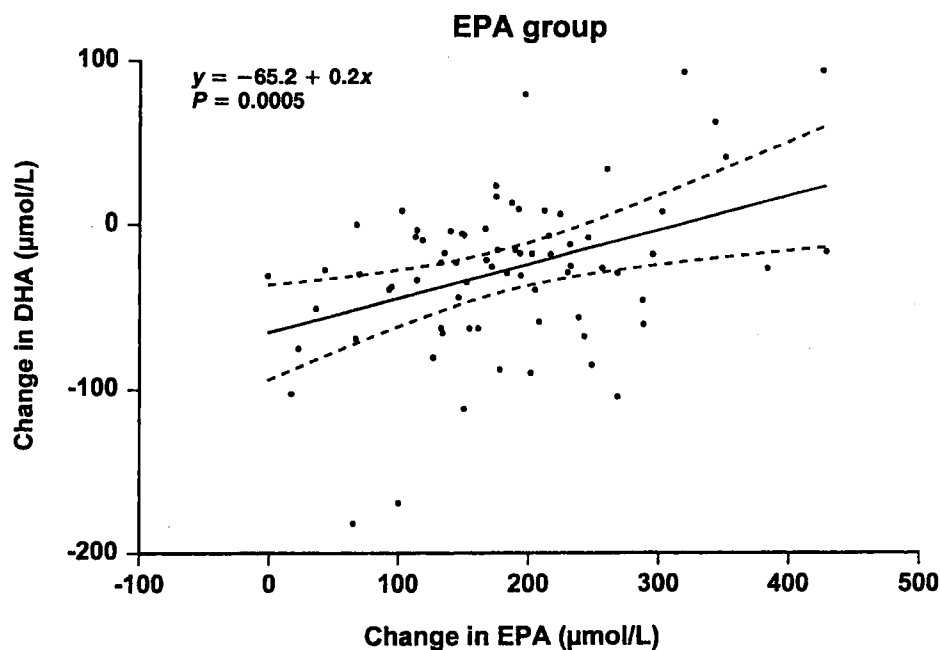


FIGURE 2. Plot of change in serum phospholipid eicosapentaenoic acid (EPA) concentration versus change in serum phospholipid docosahexaenoic acid (DHA) concentration in the EPA supplementation group ($n = 75$).

oil supplementation produced a rapid increase in plasma EPA that soon reached a plateau, whereas DHA concentration increased slowly and progressively (33). One might speculate that ingested EPA is more readily absorbed than DHA. Nevertheless, the observed ratio of DHA to EPA in serum phospholipids at baseline exceeded that of dietary intake (3.8 compared with 2.5, $P = 0.0001$).

In a metabolic study comparing ingestion of highly purified DHA and EPA, the two fatty acids produced identical increases in plasma chylomicron n-3 concentrations, but DHA was more rapidly cleared from plasma than was EPA (JB Hansen, personal communication, 1996). DHA is known to accumulate in the central nervous system and in cardiac tissue, and advanced atherosclerotic plaques are enriched with more DHA than EPA after dietary supplementation (42). These results indicate that the two fatty acids are distributed into different compartments of the body and have different metabolic actions. The present study supports the proposed pattern of incorporation in which DHA is selectively incorporated into extracirculatory pools whereas EPA has priority in the circulatory pool.

In the DHA group, the content of both DHA and EPA increased in serum phospholipids, indicating retroconversion of DHA to EPA. The degree of retroconversion appeared to be modest and may reach a saturation point (Figure 1). Similar findings were reported in cell and animal studies (11, 31) as well as in humans after dietary supplementation with highly purified EPA and DHA (10, 15, 16, 43). Theoretically, the increase of serum EPA in the DHA group could result from the small amount of EPA present in the DHA supplements (0.072 g/d). To examine this possibility, we did a regression analysis with dietary intake of EPA as the predictor variable and baseline serum phospholipid EPA concentration as the dependent variable (serum phospholipid EPA = $46.6 + 81.5$ multiplied by dietary intake; $F = 0.0001$). The model predicted that the amount of extra EPA provided by the DHA supplement would increase serum phospholipid EPA to 67.8 mmol/L (95% CI: 60.4, 75.2) in the DHA group. The concentration of EPA after DHA supplementation (77.4 mmol/L) was higher, however, suggesting that retroconversion of DHA to EPA took place. Hence, ingested DHA may serve as a reservoir for EPA.

In the EPA group, there was a substantial increase in serum EPA and DPA whereas DHA decreased. This finding suggests that purified EPA is elongated to DPA, but not to DHA, and confirms previous studies in both rats and humans (10, 15, 16, 31). It is possible that EPA displaces DHA from the 2-position in serum phospholipids. Nevertheless, DHA concentrations increased modestly (20%) in 14 participants in the EPA group (Figure 2). This subgroup was characterized by large increases in both EPA (360%) and DPA (189%) during the intervention. Although not detectable from their dietary habit records, it may be that these individuals increased their fish consumption and consequently their DHA concentrations increased during intervention. An alternative explanation is that some DHA production takes place when EPA and DPA concentrations increase substantially.

A recent report established that DHA is biosynthesized from α -linolenic acid in human infants, although the conversion rate may be inadequate to support infant needs (44). Thus, α -linolenic acid and EPA may have different meta-

bolic pathways because it seems that humans are not capable of synthesizing DHA from EPA unless the EPA concentration is high. This may have clinical importance if the purpose of n-3 fatty acid supplementation is to increase DHA concentration.

The change in the ratio of n-3 to n-6 serum phospholipid fatty acids was larger in the EPA group than in the DHA group. The proposed beneficial effects of n-3 fatty acids on cardiovascular disease have been partly attributed to an increase in the ratio of n-3 to n-6 fatty acids in cell membranes, resulting in a shift in eicosanoid synthesis toward a more vasodilatory and antiaggregatory state (5). In this study, the arachidonic acid concentration decreased similarly in the EPA and DHA groups. In the DHA group, however, the ratios (ie, desaturation indexes) of the individual n-6 fatty acids changed more than in the EPA group. Bearing in mind that serum phospholipid fatty acids are influenced by several factors, one might speculate that EPA and DHA have different effects on liver desaturation enzymes. Apparently, EPA exerts no effects on liver desaturation activity, suggesting that arachidonic acid decreased as a result of displacement from serum phospholipids. In the DHA group however, the decrease in arachidonic acid concentration may have resulted from decreased $\Delta 6$ and $\Delta 5$ desaturation. These enzymes are influenced by several factors including diet (45). n-3 Fatty acids decrease $\Delta 6$ and $\Delta 5$ desaturation in rats (46-48) and DHA in particular has been held responsible for reducing $\Delta 6$ desaturation, possibly by a feedback mechanism (31). The present data suggest that n-3 fatty acids may influence eicosanoid synthesis both by reducing arachidonic acid synthesis (DHA) and by displacing arachidonic acid from serum phospholipids (EPA).

In both the EPA and DHA groups there was a reduction in monoenes. This may represent a reduction in $\Delta 9$ desaturation as judged by the ratios of 18:0 to 18:1n-9 and of 16:0 to 16:1n-7. Similar observations were made in rats after supplementation with n-3 fatty acids (48, 49). Hence, n-3 fatty acids may affect membrane structure and function by altering activity in $\Delta 9$, $\Delta 6$, and $\Delta 5$ desaturation enzymes.

There were no important clinical or biochemical side effects during 257 man-months (number of men taking the supplements times the number of months the supplements were consumed) of dietary supplementation with highly purified ethyl esters of EPA or DHA, although transient belching with a taste of fish oil was experienced frequently. Consequently, participants knew they were ingesting an n-3 fatty acid but not whether it was EPA or DHA. This points to a blinding problem in controlled studies with n-3 fatty acids. Compliance was slightly poorer and side effects a little more frequent in the DHA group.

We conclude that both DHA and EPA lower serum triacylglycerol concentration, but have differential effects on lipoprotein and fatty acid metabolism in humans. The present data suggest that effects of individual n-3 fatty acids should be taken into consideration when interpreting the effects of n-3 fatty acid supplementation.

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Effect of eicosapentaenoic acid ethyl ester on hypothyroid function

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Abstract

Thyroid hormones affect reactions in almost all pathways of lipid metabolism. It has been reported that plasma free fatty acid (FFA) concentration in hypothyroidism is generally within the normal range. In this study, however, we show that plasma FFA concentration in some hypothyroid patients is higher than the normal range. Symptoms of thyroid dysfunction in these individuals were less severe than those of patients with lower plasma FFA concentrations. From these findings we hypothesized that the change in FFA concentration must correlate with thyroid function. Using an animal model, we then examined the

effect of highly purified eicosapentaenoic acid ethyl ester (EPA-E), a n-3 polyunsaturated fatty acid derived from fish oil, on thyroid function in 1-methyl-2-imidazoethiol (MMI)-induced hypothyroid rats. Oral administration of EPA-E inhibited reduction of thyroid hormone levels and the change of thyroid follicles in MMI-induced hypothyroid rats. These findings suggest that FFA may affect thyroid functions and EPA-E may prevent MMI-induced hypothyroidism.

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Introduction

Thyroid hormones affect reactions in almost all pathways of lipid metabolism (Hoch 1988). Plasma free fatty acid (FFA) is released from triglyceride-rich lipoprotein by lipoprotein lipase and hepatic triglyceride lipase. Although it has been reported that plasma lipoprotein lipase and hepatic triglyceride lipase activities are increased in hypothyroidism (Valdemarsson 1983), several studies have shown that plasma FFA concentration in hypothyroidism is within the normal range (Hamberger *et al.* 1963, Tulloch *et al.* 1973, Saunders *et al.* 1980). Therefore, the relationship between plasma FFA concentration and the hypothyroid state remains unclear.

Fatty acids are important as an energy source and as essential components of the plasma membrane. Plasma membrane is composed of several different categories of lipids and functional proteins. Since fatty acid composition is a major factor influencing the biological functions of plasma membranes, structural and quantitative changes of fatty acids may alter the physical properties of the membrane. Fatty acids, especially polyunsaturated fatty acid (PUFA), may affect cellular functions such as membrane-bound enzymes (Neelands & Clandinin 1983), transport systems (Ekokoski *et al.* 1994) and receptors (Gould *et al.* 1982). In addition, several studies have shown that PUFAs are ligands for peroxisome proliferator activated receptor α

(PPAR α) (Forman *et al.* 1997, Kliewer *et al.* 1997). Moreover, PPAR α is related to cell proliferation and differentiation in various cells (Hanley *et al.* 1998, Kaplanski *et al.* 2000, Peters *et al.* 2000).

Eicosapentaenoic acid (EPA), an n-3 PUFA, found in various human tissues, has beneficial effects against several diseases (Kromann & Green 1980). To determine whether an increase in plasma FFA concentration might affect thyroid function, in the second part of this study we investigated whether eicosapentaenoic acid ethyl ester (EPA-E) might prevent the hypothyroid state in 1-methyl-2-imidazoethiol (MMI)-induced hypothyroid rats.

Subjects and Methods

Patients and treatment

The clinical study consisted of 15 unrelated Japanese subjects diagnosed with primary hypothyroidism on the basis of their symptoms and laboratory tests at Fujita Health University Hospital. After diagnosis and having obtained their consent to our study, patients were treated by oral administration of thyroxine at an initial dose of 25 μ g daily, and subsequently at 25 μ g every 2 weeks until plasma thyroid-stimulating hormone (TSH) concentration reached normal levels.

Table 1 Clinical and biochemical characteristics of 15 unrelated Japanese subjects with hypothyroidism. The high and low FFA groups were classified according to their fasting serum FFA levels. Each value is the mean $\pm$ S.D.

	Low FFA level	High FFA level
Symptoms (%)		
Fatigue	100	100
Cold intolerance	63	29
Edema	75	43
Dry skin	75	29
Slow speech	75	29
Constipation	75	29
Delay of ATR	88	29*
Laboratory data		
GOT (mU/ml)	61.0 $\pm$ 60.5	30.9 $\pm$ 37.3
GPT (mU/ml)	63.9 $\pm$ 91.9	19.5 $\pm$ 16.6
CK (mU/ml)	331.5 $\pm$ 202.6	149.5 $\pm$ 260.4
Total cholesterol (mg/dl)	275.0 $\pm$ 95.5	261.7 $\pm$ 51.8
Triglyceride (mg/dl)	176.8 $\pm$ 39.7	126.7 $\pm$ 71.4
HDL cholesterol (mg/dl)	28.8 $\pm$ 9.3	59.3 $\pm$ 28.8
β -lipoprotein (mg/dl)	150.3 $\pm$ 188.9	498.3 $\pm$ 150.3
FFA (mEq/l)	0.17 $\pm$ 0.05	0.88 $\pm$ 0.24*
Thyroid function		
TSH (μ U/ml)	141.9 $\pm$ 43.0	65.6 $\pm$ 38.1**
T_3 (ng/ml)	0.45 $\pm$ 0.24	1.06 $\pm$ 0.46**
T_4 (μ g/dl)	1.23 $\pm$ 1.16	3.56 $\pm$ 2.47*
Free T_3 (pg/ml)	1.16 $\pm$ 0.74	2.4 $\pm$ 1.08*
Free T_4 (ng/dl)	0.18 $\pm$ 0.20	0.47 $\pm$ 0.25*

* $P < 0.05$, ** $P < 0.01$; statistically significant, compared using Fisher's exact probability test (symptoms) or Mann-Whitney U test (laboratory data and thyroid function).

Animals and manipulation

Five-week-old male Wistar rats were used in the experiments described in this study. We obtained 12 rats, weighing from 100 to 110 g, from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. The rats were divided into two groups of six on the basis of their initial body weights. Hypothyroidism in rats was induced by subcutaneous injection of MMI (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) at a daily dose of 1 mg with concomitant oral administration of EPA-E (generously donated by Mochida Pharmaceutical Co., Ltd, Tokyo, Japan) at a daily dose of 300 mg/kg (or the same volume of saline as a control) for 4 weeks. After 4 weeks the rats were anesthetized with chloroform and blood samples and thyroid tissues were obtained. Part of the thyroid tissues were immediately frozen in liquid nitrogen and stored at -80°C until use. This work conformed to the guidelines on the handling of laboratory animals of our institution.

Determination of serum tri-iodothyronine (T_3), thyroxine (T_4) and TSH

Serum levels of T_3 , T_4 and TSH (patients) and T_3 and T_4 (animals treated with and without EPA-E) were measured by an immunochemiluminescence method (Amersham Pharmacia Biotech, Little Chalfont, UK); normal range of

T_3 , T_4 and TSH concentrations are 0.9–1.5 ng/dl, 5.0–9.7 $\mu\text{g/ml}$ and 0.4–4.0 $\mu\text{g/ml}$ respectively. Serum levels of rat TSH were measured using a rat TSH (rTSH) enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech); normal range of rTSH concentration is 5–30 ng/ml.

Analysis of plasma lipid and FFA composition

At 9 weeks of age, after a 10-h fast, blood samples were taken from the tail veins of the rats without anesthesia. Plasma levels of total cholesterol, triglyceride and FFA were measured enzymatically with an autoanalyzer (Olympus Optical, Co., Ltd, Tokyo, Japan). FFAs were then separated and quantified by gas chromatography (model 663 Hitachi, Tokyo, Japan) using a capillary column (SP-2380).

Analysis of FFA composition in thyroid tissues

Thyroid tissues were obtained from the rats killed by exsanguination under anesthesia. Thyroid tissues were homogenized in a mixture of chloroform and methanol (2:1 vol./vol.), and total lipid extracts were prepared according to the method (Folch *et al.* 1957). The determination of FFA composition in the thyroid tissue extracts was performed as described for the determination of plasma FFA composition.

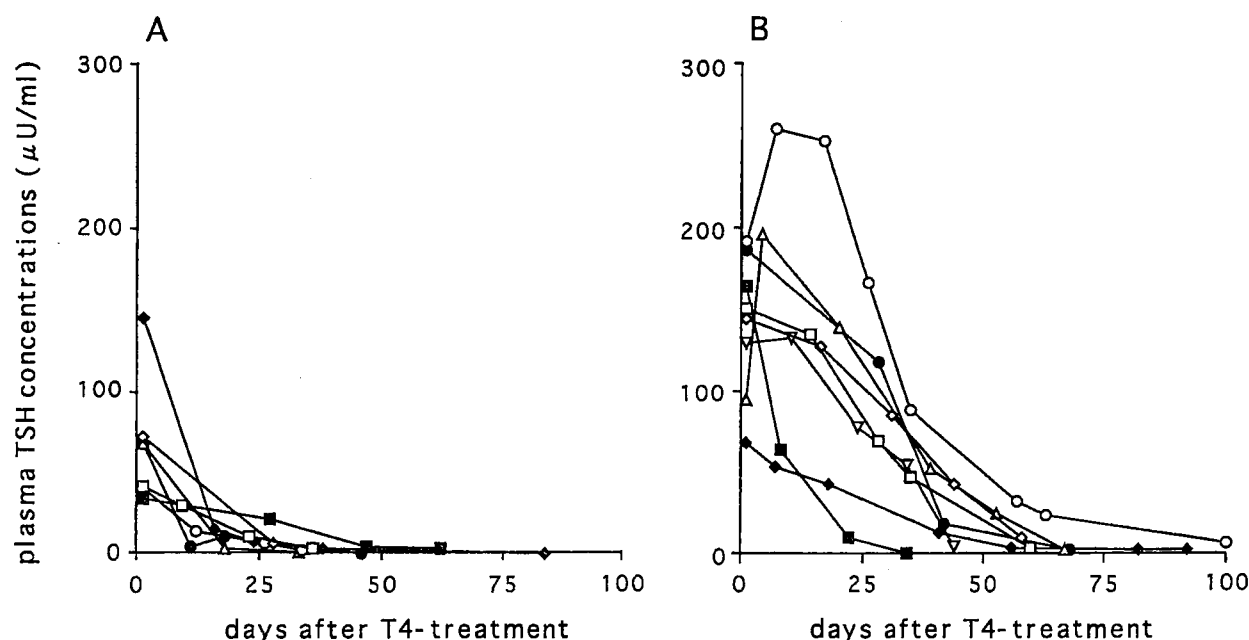


Figure 1 Change of serum TSH concentration in hypothyroid patients after T_4 treatment. (A) Seven hypothyroid patients with high plasma FFA levels. (B) Eight hypothyroid patients with low plasma FFA levels. The periods until normalization of TSH concentrations after the treatment of the patients with the high plasma FFA level were shorter than that of the patients with the low plasma FFA levels (38.1 ± 12.8 vs 62.6 ± 12.0 , $P < 0.01$, mean $\pm$ s.d.), and also the area under the curve was smaller (1031.0 ± 487.7 vs 5599.4 ± 3177.9 , $P < 0.01$, mean $\pm$ s.d.).

Histopathological examination

The portions of thyroid tissues were immediately dissected and immersed in neutralized 10% formalin for 5 days. Specimens were then embedded in paraffin by routine procedures. Paraffin sections of the specimens were deparaffinized with xylene and stained with hematoxylin and eosin for light microscopy examination.

Statistical analysis

Data are expressed as the mean $\pm$ s.d. The significance of differences versus the control group was analyzed by Fisher's exact probability test (symptoms in hypothyroidism patients) or Mann-Whitney U test (laboratory data in hypothyroidism patients and in animal study), and a statistically significant difference was defined at P less than 0.05.

Results

Clinical experiments

Clinical and biochemical characteristics of hypothyroid patients Some hypothyroid patients had a higher plasma FFA concentration than normal range. The severity of hypothyroid symptoms and abnormal values of biochemical markers was elevated in those patients with

low levels of plasma FFA (Table 1). Serum T_3 and T_4 concentrations were significantly decreased, and serum TSH concentrations were significantly increased, in patients with low plasma levels of FFA when compared with patients with high levels of plasma FFAs (Table 1).

Changes in TSH concentrations in patients with hypothyroidism after treatment Patients with high plasma levels of FFA exhibited more rapid normalization of TSH than patients with low plasma FFA (Fig. 1).

Animal experiments

Animal condition There were no significant differences in food intake and body weight of the MMI-induced hypothyroid animals during the experimental period (Table 2). Thyroid weight was, however, noticeably greater in animals treated without EPA-E than in animals treated with EPA-E (Table 2).

Lipid levels in thyroid tissues and plasma in hypothyroid rats Plasma FFA levels showed no statistically significant differences between animals treated with and without EPA-E during the experimental period (Table 2), nor was any significant difference observed between the groups in total cholesterol and triglyceride levels (Table 2). MMI-induced hypothyroid rats treated with EPA-E

Table 2 The change of animal condition and lipid for rats with MMI-induced hypothyroidism with or without EPA. Each value is the mean $\pm$ S.D.

	Treatment without EPA-E (days)					Treatment with EPA-E (days)				
	0	7	14	21	28	0	7	14	21	28
Body weight (g)	145 ± 9	221 ± 13	231 ± 12	252 ± 9	272 ± 8	146 ± 3	220 ± 14	243 ± 16	269 ± 17	283 ± 9
Heart rate (beats/min)	445 ± 33		418 ± 23		439 ± 21	457 ± 43		444 ± 36		445 ± 33
Thyroid weight (mg)					36.5 ± 5.6					29.7 ± 1.9*
Heart weight (mg)					980.3 ± 90					930.2 ± 77.3
Total cholesterol (mg/dl)					61.75 ± 2.63					65.75 ± 5.56
Triglyceride (mg/dl)					26 ± 6.98					25.75 ± 15.65
FFA (mEq/l)	1.898 ± 0.27				0.582 ± 0.161	1.754 ± 0.244				0.662 ± 0.26

* $P < 0.05$; statistically significant, compared using Mann-Whitney U test.

showed elevated level of EPA (C20:5) in plasma and thyroid tissues compared with hypothyroid rats treated without EPA-E (Table 3).

Changes in serum T_3 , T_4 and rTSH levels in MMI-induced hypothyroid rats Hypothyroid rats treated with EPA-E exhibited significantly higher levels of serum T_3 and T_4 throughout the experimental period than rats treated with EPA-E (Fig. 2).

After these results were obtained, this experiment was reconfirmed by the same methods using seven rats from each group. Serum T_3 , T_4 and rTSH levels of rats treated with EPA-E versus rats treated without EPA-E were 0.82 ± 0.08 vs 0.72 ± 0.06 mg/ml ($P=0.0181$), 4.63 ± 0.38 vs 4.10 ± 0.34 μ g/dl ($P=0.0253$), and 34.58 ± 8.43 vs 62.26 ± 11.40 ng/ml ($P=0.017$) respectively.

In addition, 5-week-old male Wistar rats were orally administrated with EPA-E at a daily dose of 300 mg/kg ($n=4$) or the same volume of saline as a control ($n=4$) for 4 weeks. The rTSH levels in rats treated with EPA-E were not significantly different from rats treated with saline: 9.58 ± 6.36 vs 13.76 ± 6.88 ng/ml.

Light microscopic findings Histopathological features in the thyroid tissues of hypothyroid rats are shown in Fig. 3. Although the size of the thyroid follicular lumens varied in MMI-induced hypothyroid rats treated without EPA-E (Fig. 3A), the size of the thyroid follicular lumens were uniform in MMI-induced hypothyroid rats treated with EPA-E (Fig. 3B).

Discussion

While several studies have shown that plasma FFA concentration in hypothyroidism is within normal range (Hamberger *et al.* 1963, Tulloch *et al.* 1973, Saunders *et al.* 1980), some hypothyroid patients have higher plasma FFA concentrations than the normal range in our investigation. In the clinical part of our study, we confirmed that symptoms of thyroid dysfunction in hypothyroid patients with high plasma FFA concentrations were less severe than those of patients with low plasma FFA concentration. Severely hypothyroid patients had lower FFA concentrations in this study, correlating with the fact that activities of lipoprotein lipase and hepatic triglyceride lipase, key enzymes of regulation in plasma FFA, are increased in hypothyroidism (Valdemarsson 1983). Hypothyroidism in patients with high plasma FFA concentrations was less severe, therefore we hypothesized that the increase of plasma FFA concentration might affect thyroid function in the hypothyroid state. To test our hypothesis, we examined the influence of the n-3 PUFA EPA-E on rats with MMI-induced hypothyroidism. In the animal experiments, the decrease in plasma T_3 and T_4 concentrations and increase in plasma TSH concentration

Table 3 Changes in serum and thyroid FFA compositions for rats with MMI-induced hypothyroidism with or without EPA. Each value is the mean $\pm$ s.d.

	Plasma		Thyroid	
	EPA-E (-)	EPA-E (+)	EPA-E (-)	EPA-E (+)
C14:0	0.32 $\pm$ 0.05	0.37 $\pm$ 0.09	1.85 $\pm$ 0.30	2.00 $\pm$ 0.21
C16:0	18.92 $\pm$ 0.71	19.60 $\pm$ 1.32	26.77 $\pm$ 1.04	28.077 $\pm$ 0.83
C16:1	0.82 $\pm$ 0.17	1.27 $\pm$ 0.17*	2.95 $\pm$ 0.58	3.75 $\pm$ 0.41
C18:0	14.25 $\pm$ 1.66	13.32 $\pm$ 1.22	9.45 $\pm$ 0.83	7.75 $\pm$ 0.75
C18:1	6.90 $\pm$ 0.82	6.97 $\pm$ 0.69	24.30 $\pm$ 2.61	24.6 $\pm$ 0.79
C18:2	20.67 $\pm$ 1.05	21.02 $\pm$ 1.43	24.22 $\pm$ 2.13	24.47 $\pm$ 2.30
C18:3	0.35 $\pm$ 0.05	0.35 $\pm$ 0.05	0.75 $\pm$ 0.12	0.95 $\pm$ 0.17*
C20:0	0.12 $\pm$ 0.05	0.10 $\pm$ 0.00	0.17 $\pm$ 0.05	0.20 $\pm$ 0.00
C20:1	0.12 $\pm$ 0.05	0.10 $\pm$ 0.00	0.52 $\pm$ 0.05	0.50 $\pm$ 0.00
C20:2	0.22 $\pm$ 0.00	0.22 $\pm$ 0.00	0.30 $\pm$ 0.00	0.32 $\pm$ 0.05
C20:3	0.77 $\pm$ 0.09	1.1 $\pm$ 0.18	0.42 $\pm$ 0.12	0.35 $\pm$ 0.05
C20:4	29.02 $\pm$ 1.61	25.17 $\pm$ 3.52	4.25 $\pm$ 1.33	2.87 $\pm$ 0.33
C20:5	1.15 $\pm$ 0.17	3.22 $\pm$ 0.66**	0.20 $\pm$ 0.08	0.62 $\pm$ 0.09**
C22:0	0.17 $\pm$ 0.05	0.25 $\pm$ 0.05	0.17 $\pm$ 0.05	0.22 $\pm$ 0.05
C22:1	ND	ND	ND	ND
C22:5	0.85 $\pm$ 0.20	1.77 $\pm$ 0.69	0.92 $\pm$ 0.15	1.55 $\pm$ 0.28
C22:6	5.05 $\pm$ 0.26	4.80 $\pm$ 0.76	2.50 $\pm$ 1.08	1.60 $\pm$ 0.29
C24:0	0.10 $\pm$ 0.00	0.17 $\pm$ 0.15	0.01 $\pm$ 0.00	0.17 $\pm$ 0.00
C24:1	0.10 $\pm$ 0.00	0.10 $\pm$ 0.00	0.05 $\pm$ 0.00	0.10 $\pm$ 0.00

* $P < 0.05$, ** $P < 0.01$; statistically significant, compared using Mann-Whitney U test.

in the MMI-induced hypothyroid state was inhibited by administration of EPA-E. In addition, the sizes of the thyroid follicles without administration of EPA-E were varied and contained large of quantities of colloid. These findings indicate that thyroid hormone secretion in the rat in the absence of EPA-E administration is elevated.

The regulation of cell functions by PUFA, including EPA, might occur on two general levels: modulation of signal transduction via manipulation of membrane fatty acid composition; and rapid, direct modulation of gene transcription. Several studies have provided evidence indicating that plasma membrane lipids in thyroid glands

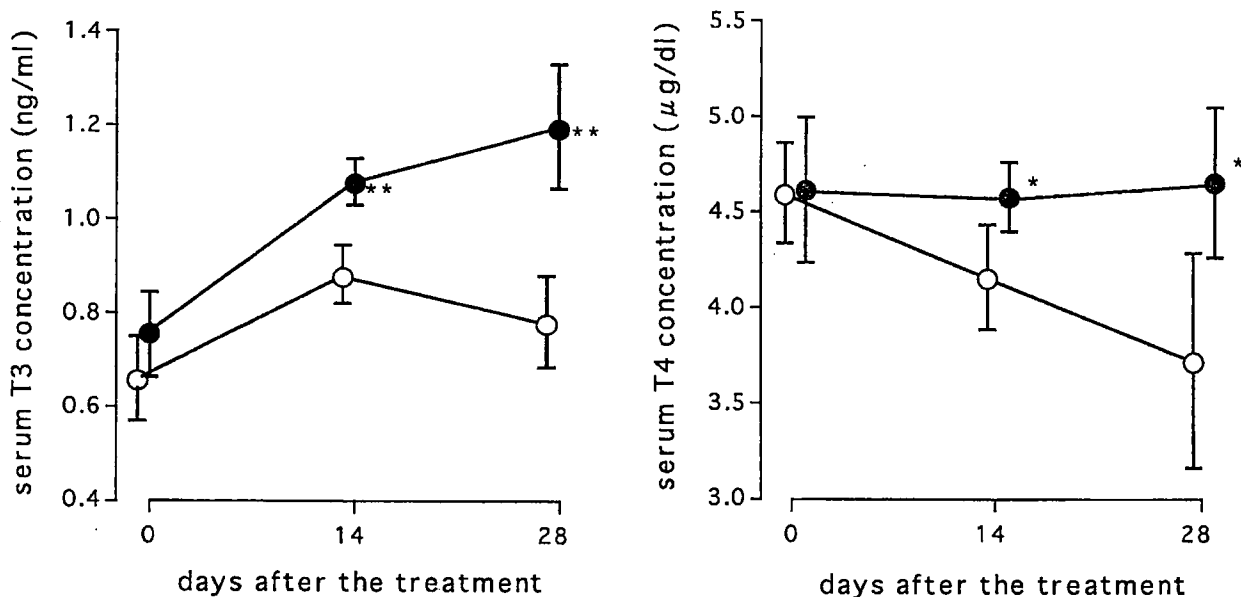


Figure 2 Changes in serum T_3 (ng/dl) and T_4 (μ g/ml) concentration for MMI-induced hypothyroid rats treated with (●) or without (○) EPA-E. Each value represents the mean $\pm$ s.d. ** $P < 0.01$, * $P < 0.05$.

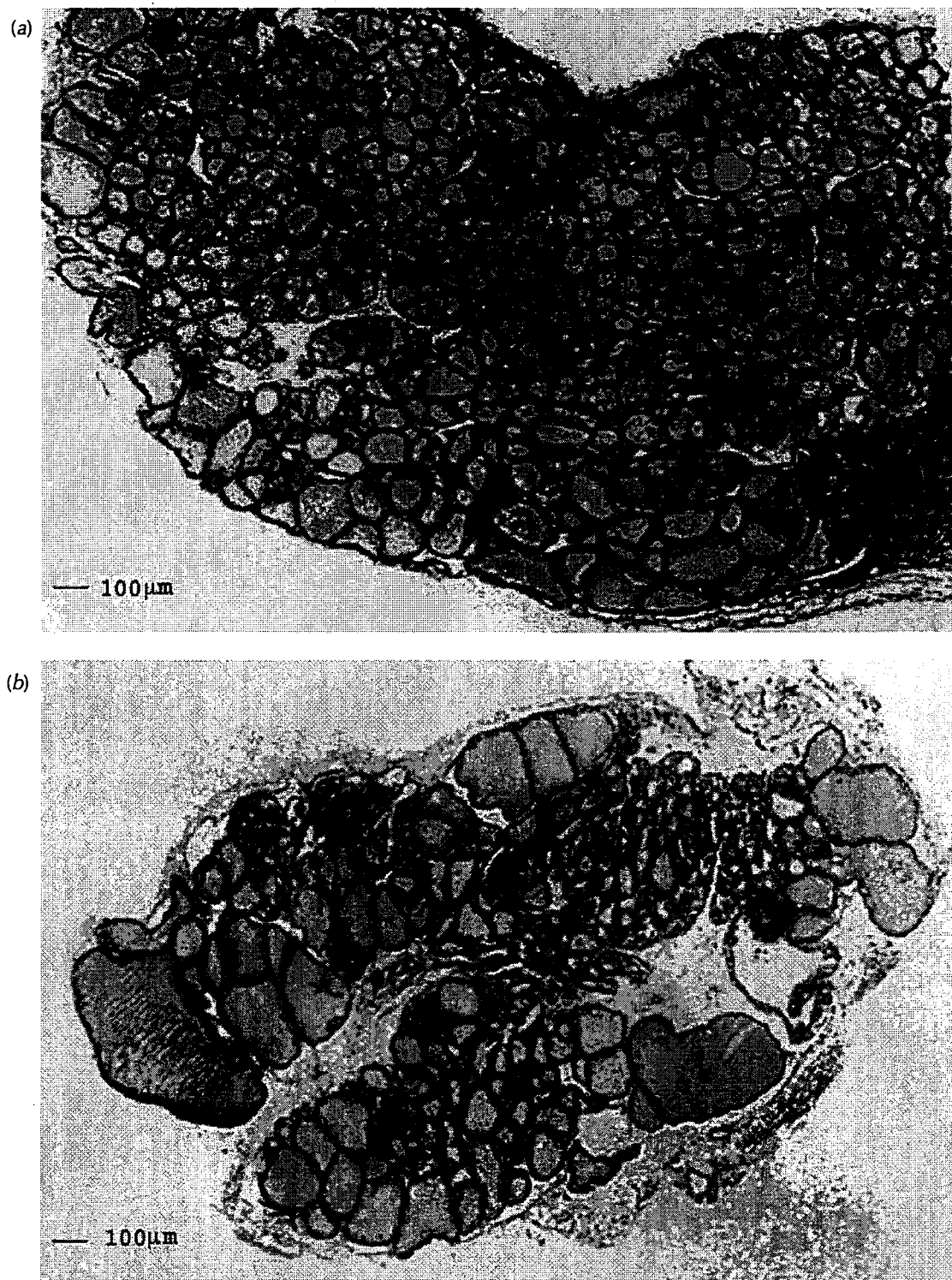


Figure 3 Histopathological analysis of the thyroid glands of MMI-induced hypothyroid rats treated with (A) or without (B) EPA-E.

modulate the TSH receptor and its ability to bind TSH (Bashford *et al.* 1975, Mehedi *et al.* 1977, Omodeo-Sale *et al.* 1978, Aloj *et al.* 1979). Furthermore, an important role for phospholipids has been indicated in the TSH-responsive adenylate cyclase system (Macchia *et al.* 1970, Yamashita *et al.* 1970, Yamashita & Field 1973, Moore & Wolff 1974, Amir *et al.* 1976).

EPA and arachidonic acid (AA), constituents of the plasma membrane, are metabolized via the cyclooxygenase, lipoxygenase or epoxygenase pathways to generate eicosanoids. Eicosanoids produced from EPA and AA have different effects in their actions (Yu *et al.* 1995). These differences may affect receptor and signal transduction, resulting in the changes of thyroid hormone biosynthesis or release.

PUFAs are ligands for PPAR α (Forman *et al.* 1997, Kliewer *et al.* 1997), a member of the nuclear receptor superfamily. PPAR α is related to cell proliferation and differentiation in hepatocytes (Peters *et al.* 2000), keratinocytes (Hanley *et al.* 1998) and oval cells (Kaplanski *et al.* 2000). Although a relationship between PPAR α and thyroid follicular cells is not apparent in cellular growth and function, it is possible that PPAR α affects thyroid follicular cell proliferation and differentiation.

It is clear that administration of EPA-E inhibits the decrease in thyroid hormone levels. It will be interesting to determine whether other PUFAs, such as AA and docosapentaenoic acid, have the same effect. Our findings suggest that FFA may affect thyroid functions, and that EPA-E may prevent MMI-induced hypothyroidism.

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The Nuclear Receptors Peroxisome Proliferator-activated Receptor α and Rev-erb α Mediate the Species-specific Regulation of Apolipoprotein A-I Expression by Fibrates*

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Fibrates are widely used hypolipidemic drugs which activate the nuclear peroxisome proliferator-activated receptor (PPAR) α and thereby alter the transcription of genes controlling lipoprotein metabolism. Fibrates influence plasma high density lipoprotein and its major protein, apolipoprotein (apo) A-I, in an opposite manner in man (increase) *versus* rodents (decrease). In the present study we studied the molecular mechanisms of this species-specific regulation of apoA-I expression by fibrates. In primary rat and human hepatocytes fenofibric acid, respectively, decreased and increased apoA-I mRNA levels. The absence of induction of rat apoA-I gene expression by fibrates is due to 3 nucleotide differences between the rat and the human apoA-I promoter A site, rendering a positive PPAR-response element in the human apoA-I promoter nonfunctional in rats. In contrast, rat, but not human, apoA-I transcription is repressed by the nuclear receptor Rev-erb α , which binds to a negative response element adjacent to the TATA box of the rat apoA-I promoter. In rats fibrates increase liver Rev-erb α mRNA levels >10-fold. In conclusion, the opposite regulation of rat and human apoA-I gene expression by fibrates is linked to differences in *cis*-elements in their respective promoters leading to repression by Rev-erb α of rat apoA-I and activation by PPAR α of human apoA-I. Finally, Rev-erb α is identified as a novel fibrate target gene, suggesting a role for this nuclear receptor in lipid and lipoprotein metabolism.

Fibrates are a class of drugs used in the treatment of atherogenic dyslipidemia (1). Fibrates are ligands for transcription factors belonging to the peroxisome proliferator-activated receptor (PPAR)¹ subfamily of nuclear receptors (2, 3). After ligand activation and heterodimerization to the 9-*cis* retinoic acid receptor RXR, PPARs bind to specific response elements, termed peroxisome proliferator response elements (PPREs), in

the regulatory regions of target genes. PPREs consist of a direct repeat of the degenerated hexamer AGGTCA sequence separated by 1 nucleotide (DR-1). Fibrates exert their effects on plasma lipids by altering the transcription of genes involved in lipoprotein metabolism (1). Direct evidence that fibrate action on lipoprotein metabolism is mediated by PPAR α , the principal PPAR form in liver, came from studies in mice rendered deficient for PPAR α by homologous recombination (4). Functional PPREs have been identified in the promoters of a number of genes involved in plasma triglyceride (e.g. human apoC-III (5) and mouse and human lipoprotein lipase (LPL) (6)) and high density lipoprotein (HDL) metabolism (e.g. human apoA-I (7) and apoA-II (8)).

Whereas both in man and rodents fibrates efficiently decrease plasma triglycerides, their effects on HDL occur in an opposite manner in these species. Whereas in man plasma HDL cholesterol consistently increases upon fibrate treatment (9), a pronounced decrease is observed in rats (10). Previously, it was demonstrated that these species differences in HDL response to fibrates are associated with opposite changes of apoA-I expression, the major HDL apolipoprotein. In rats, fibrate treatment lowers hepatic, but not intestinal, apoA-I mRNA levels, due to a decreased transcription rate of the apoA-I gene in liver (10). In contrast, the transcription rate of the human apoA-I gene is induced by PPAR α , which interacts with a positive PPRE located in the A site of the human apoA-I gene promoter liver-specific enhancer (7). Furthermore, fibrates enhance apoA-I expression and production both *in vivo* in man and *in vitro* in human hepatoma cells (11–14). The observation that fibrates regulate human and mouse apoA-I gene transcription in an opposite manner in livers of transgenic mice overexpressing the human apoA-I gene driven by its homologous promoter including the PPRE sequence (15) suggested that the species-specific differences in fibrate response are due to *cis*-element sequence differences between the rodent and human apoA-I genes. However, the exact molecular mechanisms implicated have not been determined yet. In addition, although the mechanisms of positive regulation of gene expression by fibrates via PPAR interacting with positive PPRE sequences are well understood (for review, see Schoonjans *et al.* (16)), little is known on the mechanisms of negative gene regulation by fibrates and PPARs.

In this study, we determined the molecular mechanisms behind the differential regulation of rat *versus* human apoA-I gene expression by fibrates. Our results demonstrate that this opposite regulation is due to sequence differences of two distinct enhancer regions in the rat and human apoA-I genes. On the one hand, the human apoA-I gene promoter A site contains a positive PPRE to which PPAR α binds. The lack of induction of apoA-I gene transcription by fibrates in rats is due to 3

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¹ The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, 9-*cis* retinoic acid receptor; RevRE, Rev-erb α response element; TK, thymidine kinase; HDL, high density lipoprotein; TR, thyroid hormone receptor; EMSA, electrophoretic mobility shift assays; TRE, TR response element; CAT, chloramphenicol acetyltransferase.

nucleotide differences between the rat and the human apoA-I gene promoter A sites, resulting in the transformation of the positive PPRE in the human apoA-I promoter in a nonfunctional site in rats. On the other hand, rat, but not human, apoA-I gene transcription is repressed via an element overlapping the TATA box to which the nuclear orphan receptor Rev-erb α binds. Furthermore, fibrates induce Rev-erb α and repress apoA-I gene expression only in rat liver, but not in intestine. Interestingly, the rat apoA-I promoter Rev-erb α response element (RevRE) is not conserved in the human apoA-I gene promoter.

EXPERIMENTAL PROCEDURES

Materials—Fenofibric acid and fenofibrate, and ciprofibrate were kind gifts of Dr. Alan Edgar (Laboratoires Fournier, Daix, France) and Dr. Herbert (Sanofi, Toulouse, France) respectively. Bezafibrate and hematoxylin were from Sigma.

Animal Studies—Adult Sprague-Dawley rats (3 months old) were treated for 14 days with the specified fibrates at the indicated concentrations (w/w) mixed in chow. At the end of the treatment period all animals were fasted overnight, weighed, and sacrificed by exsanguination under ether anesthesia between 9 and 11 a.m. Livers were frozen in liquid N₂ or stored in phosphate-buffered saline until RNA or *in situ* hybridization analysis.

Cell Culture—Human hepatoma HepG2 cells (E.C.A.C.C., Porton Down, Salisbury, UK) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO₂, 95% air. Medium was changed every other day. Human and rat hepatocytes were isolated by collagenase perfusion and cultured as described previously (17). At the end of the treatment period with fenofibric acid (in Me₂SO), cells were washed three times with ice-cold phosphate-buffered saline, homogenized in 4 M guanidinium isothiocyanate, and used for RNA analysis.

RNA Analysis—RNA preparation, Northern blot hybridizations, and quantifications were performed as described previously (10). The rat apoA-I (10), apoE (10), Rev-erb α (18), HNF-4 (19), and human acidic ribosomal phosphoprotein 36B4 cDNAs (20) were used as probes.

Recombinant Plasmids—Polymerase chain reaction amplification and cloning of the rat apoA-I promoter into the pBLCAT5 promoterless expression vector was described previously (21). Site-directed mutagenesis of the apoA-I RevRE was accomplished using the oligonucleotide 5'-CAC ACA TAT ATA GGC AGG GAA GAA GA-3' as a mutagenic primer on single-stranded DNA templates according to Kunkel (22). The rat wild-type (5'-GAT CCA CAC ATA TAT AGG TCA GGG AAG AAG A-3') and mutant apoA-I RevRE (5'-GAT CCA CAC ATA TAT AGG CAG GGA AGA AGA-3') and the rat (5'-GAT CCA CTG AAC CCT TGA TCC CAG CTC TG-3') and human wild type (5'-GAT CCA CTG AAC CCT TGA CCC CTG CCC TA-3') and mutant (5'-GAT CCA CTG ATC CCT TGT CCC CTG CCC TA-3') apoA-I A-site oligonucleotides were cloned into the *Bam*HI/*Bgl*II sites of pIC20H (23), digested with *Hind*III, and subcloned upstream of the thymidine kinase (TK) promoter of pBLCAT4 (24) to generate, respectively, rAI-TATA_{wt}-TKCAT, rAI-TATA_{mt}-TKCAT, rAI-A_{wt}-TKCAT, hAI-A_{wt}-TKCAT, and hAI-A_{mt}-TKCAT. Identity of all clones was verified by sequence analysis.

Transient Transfections—Transfections in HepG2 cells were performed at 50–60% confluency by the calcium phosphate coprecipitation procedure with a mixture of plasmids that contained in addition to the reporter (1–5 μ g/60 mm culture dish) and expression vector(s) (100–1000 ng), 0.35 μ g of cytomegalovirus- β -galactosidase expression vector as a control for transfection efficiency. All samples were complemented to an equal total amount of DNA. After 4 h cells were washed with phosphate-buffered saline and incubated for another 16 h with the indicated stimuli in fresh medium containing 5% calf serum delipoproproteinized by ultracentrifugation in KBr (1.21 g/ml) and subsequently treated with AG-1-X8 resin (Bio-Rad) plus activated charcoal. Stimuli were dissolved in Me₂SO and added to the medium at the indicated concentrations and periods of time. Control cells received vehicle only. CAT activity was determined on cell extracts as described by Gorman *et al.* (25). Autoradiographs were quantified by liquid scintillation counting and results were normalized for transfection efficiency. Transfection experiments were performed at least three times.

Electrophoretic Mobility Shift Assays (EMSA)—hPPAR α , mRXR α , and hRev-erb α proteins were synthesized *in vitro* using the rabbit reticulocyte lysate system (Promega). EMSAs with Rev-erb α , PPAR and/or RXR were performed as described previously (7, 26). For competition experiments, increasing amounts of the indicated cold oligonu-

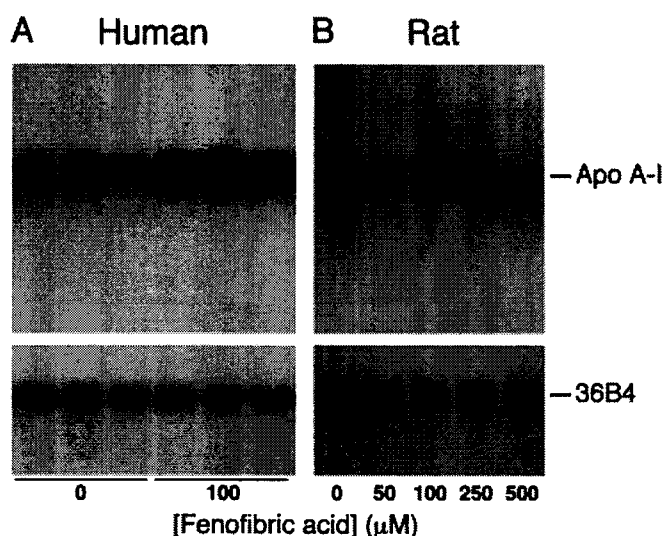


FIG. 1. Fenofibric acid influences apoA-I mRNA levels in primary cultures of adult human and rat hepatocytes in an opposite manner. Human (A) and rat (B) hepatocytes were treated for 24 h with fenofibric acid at the indicated doses. Total RNA (10 μ g) was subjected to Northern blot analysis using human (A) or rat (B) apoA-I (top panels) and 36B4 (bottom panels) cDNA probes.

cleotides were included just before adding labeled oligonucleotide. After 15 min of incubation at room temperature, DNA-protein complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.25 M Tris borate-EDTA buffer at 4 °C.

RESULTS

ApoA-I Gene Expression Is Regulated in an Opposite Manner by Fenofibrate in Human Versus Rat Hepatocytes—To study whether the species differences in response to fibrates are due to a distinct regulation of apoA-I expression in liver, the effects of fenofibric acid, the active form of fenofibrate, on apoA-I gene expression were compared between primary cultures of human versus rat hepatocytes. Addition of fenofibric acid to primary human hepatocytes, at a concentration (100 μ M) within the range of plasma concentrations attained in fenofibrate-treated patients, resulted in a >2-fold increase of apoA-I mRNA levels (Fig. 1A). In contrast, in rat hepatocytes fenofibric acid decreased apoA-I mRNA levels in a dose-dependent manner (Fig. 1B). As a control, mRNA levels of the ribosomal protein 36B4 did not change upon treatment of either human or rat hepatocytes with fenofibric acid (Fig. 1). These data indicate that the species-distinct regulation of the apoA-I gene is due to a direct effect of fenofibrate on the hepatocyte.

The Human, but Not the Rat, ApoA-I Promoter A Site Contains a Positive PPAR-response Element—Since previously a positive PPRE has been identified in the human apoA-I promoter A site (7), we wondered whether the lack of induction of apoA-I gene expression by fibrates in rats could be due to the absence of a functional PPRE in the rat apoA-I promoter. Interestingly, when the human and rat apoA-I A sites, which are composed of 3 degenerated hexameric half-sites separated by 2 and 1 nucleotides, respectively (DR-2 and DR-1), were aligned, 3 nucleotide differences were observed (Fig. 2A). To determine whether these nucleotide differences conferred a different PPAR binding potential to the rat and human apoA-I A sites, EMSA experiments were performed. Incubation of radiolabeled human A site oligonucleotide with *in vitro* produced PPAR protein, in the presence of its heterodimeric partner RXR, resulted in the formation of a retarded complex (Fig. 2B, lane 3). This complex was specific since it could be competed out by adding excess unlabeled human A site oligonucleotide

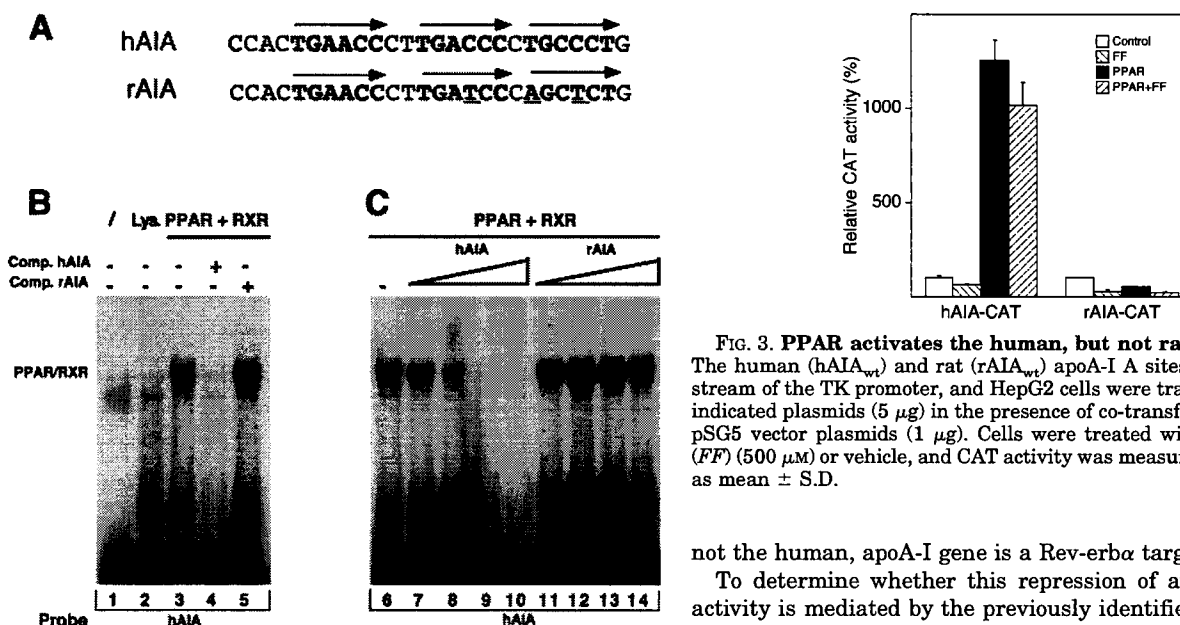


FIG. 2. PPAR/RXR heterodimers bind to the human, but not to the rat apoA-I A site. A, sequence alignment of the human (hAIA) and rat (rAIA) wild-type apoA-I A sites. The degenerated half-sites are indicated by arrows. The sequence differences between the human and rat A sites are underlined. B and C, gel retardation assays were performed on end-labeled hAIA oligonucleotide in the presence of *in vitro* transcribed/translated hPPAR α , mRXR α , or unprogrammed reticulocyte lysate (/ = no extract added; Lys. = unprogrammed lysate). Competition experiments for binding to *in vitro* transcribed/translated hPPAR α /mRXR α were performed by adding 50 (lanes 7 and 11), 100 (lanes 4, 5, 8, and 12), 200 (lanes 9 and 13) or 400-fold (lanes 10 and 14) molar excess of cold hAIA (lanes 4 and 7–10) or rAIA (lanes 5 and 11–14) oligonucleotide.

(Fig. 2, B, lane 4, and C, lanes 7–10). In contrast, unlabeled rat A site oligonucleotide did not compete for binding to the human A site (Fig. 2, B, lane 5, and C, lanes 11–14).

Next, the human and rat A sites were cloned in front of a heterologous promoter, and transient co-transfection experiments were performed in HepG2 cells. PPAR co-transfection resulted in a significant increase of human A site driven CAT activity (Fig. 3). Due to the high basal activity of PPAR in HepG2 cells, possibly due to the activation by endogenous PPAR ligands, no further activation was observed in the presence of fenofibric acid (Fig. 3). However, activation by fibrates was observed when the same experiments were performed in other cells, such as in HeLa or CV-1 cells (not shown). In contrast, rat apoA-I A site-driven CAT activity was not increased by PPAR, fenofibric acid, or both together (Fig. 3). These data clearly indicate that only the human, but not the rat, apoA-I A site functions as a PPARE.

The Orphan Receptor Rev-erb α Represses Transcription of the Rat ApoA-I Promoter—In a previous report, the nuclear receptor ROR α 1 was identified as a positive regulator of apoA-I expression in the intestine (28). Interestingly, nuclear receptors of the ROR and Rev-erb groups regulate transcription through identical response elements, leading to cross-talk between the positive ROR and the negative Rev-erb nuclear receptors (29–32). Therefore, it was tested whether Rev-erb α , which is expressed in liver (18, 33), could act as a repressor of rat apoA-I transcription. Co-transfection of CAT reporter constructs driven by the rat apoA-I promoter with a Rev-erb α expression vector resulted in dose-dependent repression of CAT activity in HepG2 cells (Fig. 4, A and B). Interestingly, human apoA-I promoter activity was not repressed by Rev-erb α co-transfection (Fig. 4A). These results indicate that the rat, but

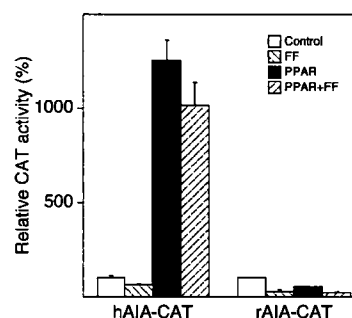


FIG. 3. PPAR activates the human, but not rat, apoA-I A site. The human (hAIA_{wt}) and rat (rAIA_{wt}) apoA-I A sites were cloned upstream of the TK promoter, and HepG2 cells were transfected with the indicated plasmids (5 μ g) in the presence of co-transfected mPPAR α or pSG5 vector plasmids (1 μ g). Cells were treated with fenofibric acid (FF) (500 μ M) or vehicle, and CAT activity was measured and expressed as mean $\pm$ S.D.

not the human, apoA-I gene is a Rev-erb α target gene.

To determine whether this repression of apoA-I promoter activity is mediated by the previously identified response element for ROR α 1 (28) located adjacent to the TATA box, the activity of Rev-erb α was analyzed on an apoA-I promoter construct carrying a mutation in the ROR α 1 response element (see Fig. 6A). Whereas wild-type apoA-I promoter activity was significantly repressed in a dose-dependent manner, Rev-erb α failed to repress the activity of the mutated construct (Fig. 4C), thereby indicating that the Rev-erb α and ROR α response elements coincide.

Rev-erb α Represses Transcription of the Rat ApoA-I Promoter through Both Active and Passive Mechanisms—Since Rev-erb α may repress transcription either actively, by recruiting nuclear co-repressors of the N-CoR family (34, 35), or passively, by competing for binding with other, positive factors, such as the proteins binding to the TATA box, the activity of a C terminus-truncated Rev-erb α 236, which can still bind to DNA, but does not actively repress transcription (36), was tested on rat apoA-I promoter transcription. In contrast to wild-type Rev-erb α , which repressed transcription already at low concentrations, Rev-erb α 236 started to repress apoA-I transcription only at higher amounts of expression vector (Fig. 5A).

To determine whether Rev-erb α could repress transcription from a heterologous promoter, the rat apoA-I TATA site (AI-TATA) was cloned in front of the TK promoter, and transient transfection assays were performed. Compared with the empty expression vector, co-transfection of Rev-erb α resulted in a significant decrease of wild-type rat AI-TATA driven CAT activity (Fig. 5B). In contrast, neither mutated AI-TATA nor TK promoter alone driven CAT activity were repressed by Rev-erb α co-transfection (Fig. 5B). Altogether, these observations suggest that the repression of rat apoA-I transcription by Rev-erb α is likely due to a combination of active and passive transcriptional repression mechanisms.

Rev-erb α Binds to the Rat, but Not the Human, ApoA-I Promoter TATA Site—EMSA were performed to determine whether Rev-erb α could bind to the rat AI-TATA site. Incubation of labeled rat wild-type AI-TATA oligonucleotide with Rev-erb α resulted in the formation of a retarded complex (Fig. 6, A and B, lane 2). The binding was specific since it could be competed out by excess amounts of either unlabeled consensus monomeric RevRE (30, 37) or rAI-TATA_{wt} oligonucleotides (Fig. 6B, lanes 3–6). In contrast, neither rAI-TATA_{mt} nor human AI-TATA oligonucleotides could compete for binding of Rev-erb α to rAI-TATA_{wt} (Fig. 6, lanes 7–10). Similar results were obtained using the consensus RevRE oligonucleotide as a probe (Fig. 6C). Similar as cold RevRE oligonucleotide (Fig. 6C,

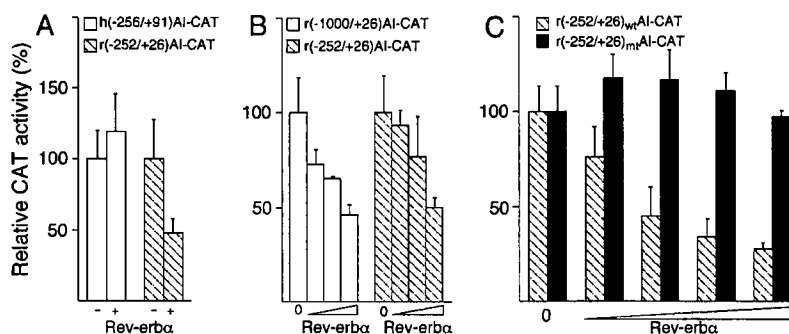


FIG. 4. **Rev-erba represses transcription of the rat, but not the human, apoA-I promoter.** A, HepG2 cells were transfected with the rat ($r(-256/+91)AI-CAT$) or human ($h(-256/+26)AI-CAT$) apoA-I promoter reporter plasmids (2 μ g) in the presence of co-transfected hRev-erba plasmid (1 μ g). B, HepG2 cells were transfected with the indicated rat apoA-I promoter reporter plasmids (2 μ g) in the presence of increasing amounts (0, 100, 500, and 1000 ng) of co-transfected hRev-erba plasmid. C, HepG2 cells were transfected with the wild-type ($r(-252/+26)AI-CAT$) or mutated ($r(-252/+26)AI-CAT$) rat apoA-I promoter constructs (2 μ g) in the presence of increasing amounts (0, 100, 250, 500, and 1000 ng) of co-transfected hRev-erba plasmid. Empty pSG5 vector was added to an equal amount of plasmid DNA. CAT activities were measured after 24 h and expressed as mean $\pm$ S.D.

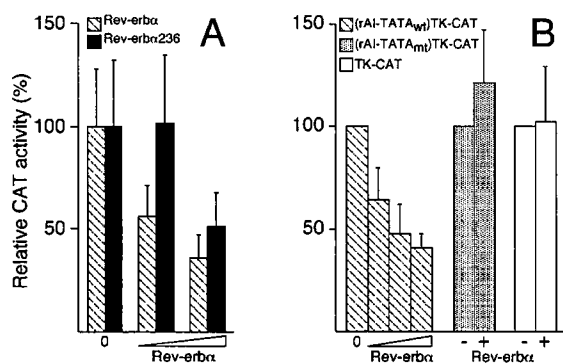


FIG. 5. **Repression of the rat apoA-I gene promoter by Rev-erba is due to both active and passive repression mechanisms.** A, HepG2 cells were transfected with the $r(-252/+26)AI-CAT$ reporter plasmid (2 μ g) in the presence of increasing amounts (0, 500, and 1000 ng) of either the wild-type (Rev-erba) or truncated (Rev-erba236) Rev-erba expression plasmids. B, HepG2 cells were transfected with the rat wild type ($rAI-TATA_{wt}-TK-CAT$) and mutated ($rAI-TATA_{mt}-TK-CAT$) apoA-I TATA site-containing TK promoter plasmids (2 μ g) in the presence of increasing amounts (0, 250, 500, and 1000 ng) of Rev-erba expression plasmid. Empty pSG5 vector was added to an equal amount of plasmid DNA.

lanes 12–14), cold $rAI-TATA_{wt}$ oligonucleotide (Fig. 6C, lanes 15–17) competed efficiently for binding of Rev-erba to RevRE, whereas the human $hAI-TATA_{wt}$ (Fig. 6C, lanes 18–20) oligonucleotide failed to compete. Altogether, these data indicate that whereas the rat apoA-I promoter contains a functional Rev-erba response element, the human AI-TATA site does not bind Rev-erba.

Fibrates Induce Rev-erba mRNA Levels in Rat Liver—To determine whether the negative regulation of rat apoA-I gene expression by fibrates is mediated via Rev-erba, adult rats were treated for 14 days with different doses of fenofibrate, and Rev-erba mRNA levels were measured by Northern blot analysis in livers and intestines, the major sites of apoA-I production. Administration of fenofibrate resulted in a pronounced induction of Rev-erba gene expression (Fig. 7A). This induction of Rev-erba mRNA was dose-dependent, being evident at a dose level of 0.05% (w/w) of fenofibrate and further enhanced at 0.5%. Interestingly, the induction of Rev-erba mRNA levels was inversely proportional to the decrease of apoA-I mRNA levels in liver (Fig. 7A). In contrast to liver, intestinal Rev-erba mRNA levels did not change significantly after fenofibrate treatment (control, $100 \pm 19\%$; fenofibrate 0.005%, $90 \pm 3\%$; fenofibrate 0.05%, $110 \pm 10\%$; fenofibrate 0.5%, $144 \pm 5\%$),

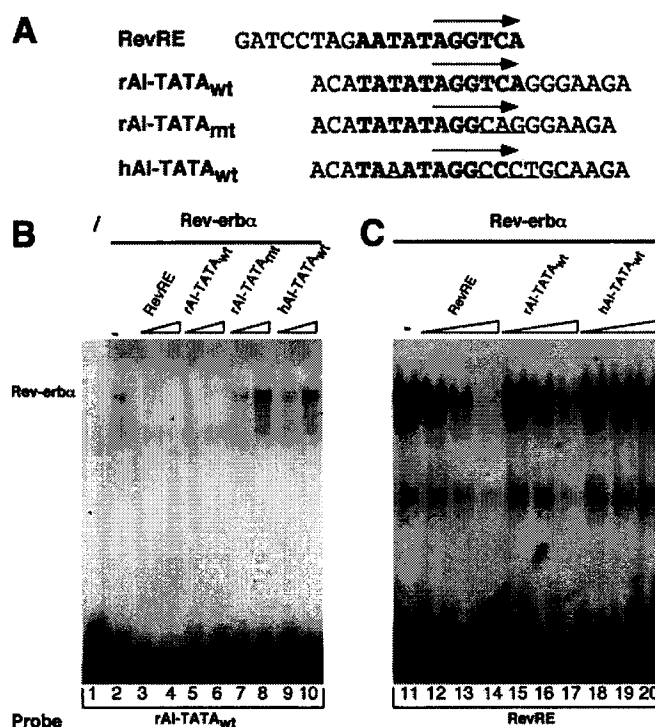
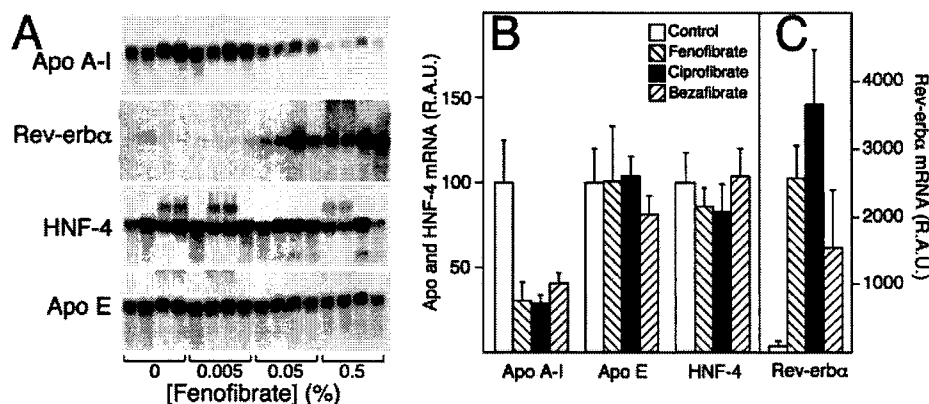


FIG. 6. **Rev-erba binds to the wild-type rat, but not to the mutated rat or human apoA-I TATA sites.** A, sequence comparison of the consensus (RevRE), the rat wild-type ($rAI-TATA_{wt}$) and mutated ($rAI-TATA_{mt}$) and the human ($hAI-TATA_{wt}$) apoA-I TATA sites. The AGGTCA half-site is indicated by an arrow. The sequence differences between the rat wild-type, mutated, and human sites are underlined. B and C, gel retardation assays were performed on end-labeled $rAI-TATA$ (lanes 1–10) or RevRE (lanes 11–20) oligonucleotides in the presence of *in vitro* transcribed/translated hRev-erba or unprogrammed reticulocyte lysate (–). Competition experiments for binding of hRev-erba were performed by adding 1 (lanes 12, 15, and 18), 10 (lanes 3, 5, 7, 9, 13, 16, and 19) and 100-fold (lanes 4, 6, 8, 10, 14, 17, and 20) molar excess of cold RevRE (lanes 3, 4, and 12–14), rat wild-type (lanes 5, 6, and 15–17), rat mutated (lanes 7 and 8) or human (lanes 9, 10, and 18–20) apoA-I TATA oligonucleotide (– = no competitor added).

which correlates well with the absence of effects of fibrates on intestinal apoA-I mRNA levels (10).

To determine whether the induction of liver Rev-erba gene expression is a general property of fibrates, rats were treated for 14 days with different fibrates. All fibrates tested significantly increased Rev-erba mRNA levels, which was most pro-

Fig. 7. Fibrates induce Rev-erb α gene expression in rat liver. Adult male rats were treated for 14 days with fenofibrate at the indicated doses (w/w in chow) (A) or with the indicated fibrates at a dose of 0.5% w/w in chow (B and C). RNA was extracted and apoA-I (A and B), HNF-4 (A and B), apoE (A and B), and Rev-erb α (A and C) mRNA levels were measured. Values are expressed relative (R.A.U. = relative arbitrary units) to chow-treated controls and represent the mean $\pm$ S.D. of four animals/group.



nounced for ciprofibrate (>30-fold), followed by fenofibrate (>25-fold) and bezafibrate ($\pm$ 15-fold) (Fig. 7B). All fibrates also repressed liver apoA-I mRNA levels (Fig. 7B). As a control mRNA levels of apoE, whose expression has been shown not to be controlled by fibrates (10), did not change significantly upon fibrate treatment (Fig. 7, A and B). Interestingly, hepatic mRNA levels of the nuclear receptor HNF-4, which is a major regulator of liver apolipoprotein gene transcription and whose expression has been shown to be repressed by fibrates (5), did not change significantly in either experiment (Fig. 7, A and B).

Finally, the induction of Rev-erb α gene expression by fibrates was also observed by *in situ* hybridization experiments using liver slices from rats treated with different fibrates (data not shown). Compared with controls, hybridization with an antisense Rev-erb α riboprobe resulted in an enhanced signal intensity in livers from fibrate-treated rats, which was evenly distributed throughout the liver, indicating that Rev-erb α mRNA levels increase after fibrate treatment in the apoA-I-producing parenchymal cells of the liver.

DISCUSSION

Fibrates are hypolipidemic drugs, which have been discovered by virtue of their cholesterol-lowering activity in rats (for review, see Staels and Auwerx (1)). Ironically, this cholesterol lowering is entirely due to a decrease of plasma HDL cholesterol concentrations and its major apolipoprotein, apoA-I (10), an effect nowadays considered to be negative, due to the protective activities of HDL and apoA-I against atherosclerosis (38, 39). In contrast, in man fibrates consistently elevate plasma HDL and apoA-I and increase the production rate of its major apolipoproteins, apoA-I and apoA-II (8, 13, 40). In the present study, we addressed the mechanisms of the differential response of apoA-I expression to fibrates in man and rat. Using primary human and rat hepatocytes we demonstrate that the opposite action of fenofibrate is due to a direct effect on the hepatocyte. These data are in line with a previous report showing opposite regulation of human (increase) *versus* mouse (decrease) apoA-I gene expression by fibrates in livers of transgenic mice overexpressing the human apoA-I gene under control of its homologous promoter (15). The experiments in these mice also indicated that this species-specific opposite regulation between human *versus* mouse is due to differences in *cis*-, but not in *trans*-acting elements (15). The results presented in this study indicate that the differences in fibrate response between the human and rat apoA-I genes are due to a combination of two distinct mechanisms implicating two different *cis*-elements binding two distinct nuclear receptors, PPAR α and Rev-erb α (Fig. 8).

On the one hand, the absence of induction of apoA-I gene expression by fibrates in rats is linked to 3-nucleotide differ-

ences between the rat and human apoA-I promoter A sites, thereby rendering the positive PPRE in the human apoA-I promoter nonfunctional in rats. Since *in vitro* promoter mapping studies identified a positive PPRE in the human apoA-I promoter A site (7), and since human apoA-I gene expression is induced at the transcriptional level by fibrate treatment in livers of transgenic mice (15) overexpressing the human apoA-I gene under control of its homologous promoter including this PPRE, it is highly likely that this element indeed confers the positive response of the human apoA-I gene to fibrates. The demonstration that this element is not functional in the rat apoA-I A site would therefore explain the lack of induction of rat apoA-I gene expression by fibrates.

On the other hand, a negative site for the nuclear receptor Rev-erb α is present in the rat, but not in the human, apoA-I promoter, and fibrate treatment results in a pronounced increase of liver expression of the Rev-erb α gene. Together with Rev-erb β (also termed RVR, BD73) and the *Drosophila* receptor E75, Rev-erb α (also termed *ear-1*) belongs to a subfamily of orphan receptors, which repress transcription of target genes (41). Rev-erb α appears to be ubiquitously expressed (18, 33), but its functions are ill defined. A number of indirect observations suggested a role for Rev-erb α in metabolic control and energy homeostasis. First, Rev-erb α expression is induced during differentiation of preadipocytes into adipocytes (42). Second, Rev-erb α is transcribed from the opposite strand of the thyroid hormone receptor (TR) α gene, with its 3' exons overlapping with TR α 2, a dominant negative regulator of TR action, suggesting a role for Rev-erb α as a modulator of thyroid hormone signaling (18, 33, 43, 44). Indeed, the level of Rev-erb α expression appears to be a determinant of the ratio of TR α 1:TR α 2 mRNA levels (42, 45, 46). In addition, Rev-erb α has been suggested to bind to a subset of TR response elements (TREs), composed of DR-4 sequences (47), as well as to compete with TR for binding to TREs (48). Finally, overexpression of Rev-erb α in mouse myoblasts leads to inhibition of muscle differentiation, possibly due to interference with thyroid hormone-induced muscle differentiation pathways (48). Interestingly, a significant level of cross-talk exists also between the peroxisome proliferator and thyroid hormone signaling pathways. Indeed, in rodents peroxisome proliferators and thyroid hormones often regulate the same target genes (49). In addition, certain of these genes have been shown to carry both TREs and PPRES (50, 51). Cross-talk between PPAR and TR may furthermore occur by competition for binding to DNA (52, 53) or for the common heterodimeric partner RXR (53–56), or by formation of PPAR:TR heterodimers, as has been suggested by one report (57). Our data identifying Rev-erb α as a fibrate target gene suggests another layer of cross-talk between the TR and PPAR

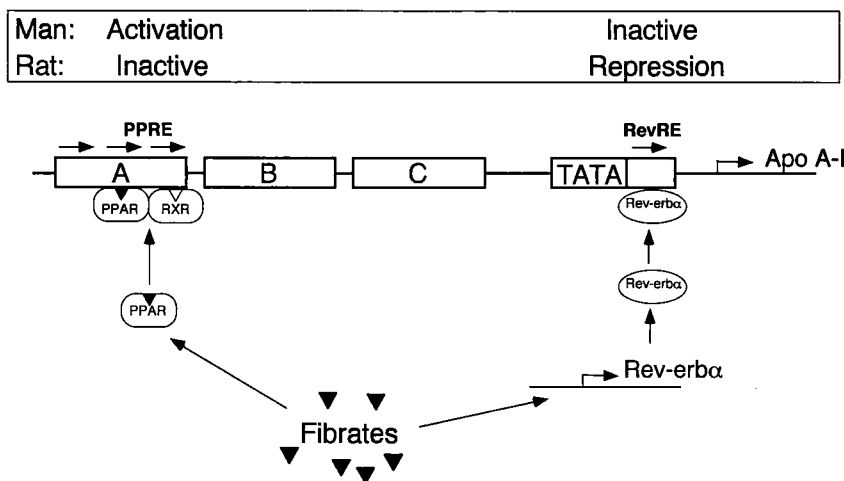


FIG. 8. Scheme summarizing our current understanding of the species-distinct regulation of apoA-I gene transcription by fibrates.

signaling pathways. Indeed it is conceivable that the induction of Rev-erb α expression after fibrates results in decreased TR α 2 mRNA levels, resulting both in a higher level of expression of functionally active TR α 1 as well as lower levels of the dominant negative TR α 2 protein, altogether leading to enhanced thyroid hormone action. Studies to test this hypothesis are currently underway in our laboratory.

The identification of the rat apoA-I gene, a major determinant of HDL metabolism, as a target for Rev-erb α , is the first demonstration of a role for this nuclear receptor in lipoprotein metabolism, albeit in rats. Although this site is not conserved in the human apoA-I gene, the fact that, both in rats (this study) and in man,² Rev-erb α expression is controlled by fibrates, which are major regulators of lipoprotein metabolism, points to a role for this nuclear receptor in lipoprotein metabolism and possibly atherogenesis. It will therefore be of interest to determine which human genes are regulated by Rev-erb α .

Rev-erb family members bind *in vitro* either to a monomeric half-site, to a subset of DR-2 (termed RevDR2) or to DR-4 TRE sequences (29–31, 36, 37, 47, 58, 59). In addition to the human Rev-erb α gene, which is negatively autoregulated by Rev-erb α binding to a RevDR2 sequence (36), the rat apoA-I gene is only the second natural Rev-erb α target gene identified to our knowledge. The Rev-erb α response element in the rat apoA-I promoter is composed of a AGGTCA half-site preceded by an AT-rich region consisting of the TATA box. Previously, it was suggested that Rev-erb α represses transcription only from RevDR2, but not from monomeric sites (31). Our results demonstrating that Rev-erb α represses transcription via a monomeric site, both in the context of the homologous as well as a heterologous promoter, suggest that Rev-erb α is also active on monomeric sites. Similarly, Rev-erb β actively represses transcription of the *N-myc* gene via a monomeric sequence (32). Active transcription repression by Rev-erb α may occur by recruiting nuclear receptor corepressors of the N-CoR family, which interact with the C terminus of Rev-erb α (34, 35). However, the truncated Rev-erb α 236 form (36), which lacks the the N-CoR binding domains, also represses transcription, albeit to a smaller extent than wild-type Rev-erb α , suggesting that the repression of apoA-I transcription by Rev-erb α is due to a combination of both active and passive mechanisms. Passive transcriptional repression may occur by competition for binding of either TATA-box binding protein (TBP) to the TATA box or for positive transcription factors to the monomeric site.

Several lines of evidence point to a functional role for Rev-erb α in the transcriptional repression of rat apoA-I gene expression by fibrates. First, the repression of rat apoA-I expression by fibrates occurs at the transcriptional level (10). Second and in contrast to the induction by fibrates of direct PPAR-target genes, such as acyl-CoA oxidase, the repression of apoA-I gene expression is a relatively slow process requiring between 12 and 24 h, pointing to an indirect action of fibrates (60). Third, the Rev-erb α response element is only present in the rat, but not in the human, apoA-I promoter, which correlates well with the difference in response of human *versus* rat apoA-I gene expression to fibrates. Finally, Rev-erb α gene expression is only induced in the liver, but not in the intestine, and fibrates repress apoA-I gene expression only in rat liver, but not in intestine (10).

In addition to the rat apoA-I gene, fibrates have been shown to repress the transcription of a wide array of genes involved in lipid and energy metabolism, such as rat S14 (56), rat apoA-IV (61), rat lecithin:cholesterol acyltransferase (62), rat hepatic lipase (63), and the human, rat, and mouse apoC-III (4, 5, 64, 65) genes in liver. Furthermore, the antidiabetic thiazolidinediones, which are ligands for PPAR γ , have been shown to repress the transcription of the human and rat leptin genes in adipose tissue (66–69). Although the mechanism of positive regulation by fibrates via PPAR interacting with PPRE sequences in target gene promoters has been fairly well studied, relatively little is known about the mechanisms of negative transcriptional regulation by these agents. Since both apoC-III and apoA-I gene expression are increased in livers of PPAR α knock-out mice and since their expression is not repressed by fibrate treatment in the same mice (4), PPAR α expression appears to be required for the negative regulation by fibrates, at least with respect to the mouse apoA-I and apoC-III genes. A previous study indicated that fibrates may repress transcription of the human and rat apoC-III and rat transferrin genes in liver by down-regulating the expression of the strong positive transcription factor HNF-4 as well as by displacement of HNF-4 binding by non-productive PPAR/RXR heterodimers (5, 70). It is unlikely, however, that the repression of rat apoA-I gene transcription occurs through this mechanism. Indeed, we could not confirm any significant regulation of HNF-4 gene expression by fibrates, whereas both Rev-erb α and apoA-I mRNA levels changed much more pronouncedly in the livers of animals treated with all fibrates tested. Moreover, whereas HNF-4 is an important regulator of hepatic apoC-III gene transcription, this transcription factor appears of lesser importance

² N. Vu-Dac, S. Chopin-Delannoy, P. Gervois, E. Bonnelye, G. Martin, J.-C. Fruchart, V. Laudet, and B. Staels, manuscript in preparation.

for human apoA-I transcriptional regulation in HepG2 cells (71, 72). In addition, the role of HNF-4 in controlling rat apoA-I gene transcription is unclear, since HNF-4 may inhibit and activate its transcription via the A and C sites, respectively (73, 74). Finally, down-regulation of HNF-4 expression and competition for its binding would be expected to affect a wide array of genes in liver whose expression is controlled by HNF-4, such as human apoA-II and apoB (75). However, whereas apoB expression is unaffected by fibrates (10), human apoA-II gene expression is induced by fibrates via PPAR:RXR binding to the HNF-4 site (8). It is tempting to speculate that the induction of Rev-erb α expression by fibrates may be a general mechanism leading to the transcriptional repression of a certain number of genes. For instance, since Rev-erb α increases upon adipocyte differentiation (42), it will be of interest to determine whether this nuclear receptor also regulates the transcription of a number of genes in adipose tissue, such as possibly the leptin gene.

In conclusion, the species-distinct regulation of apoA-I gene expression by fibrates is linked to sequence differences in cis-acting elements (see Fig. 8 for overview). In man, apoA-I transcription is induced via PPAR α binding to a positive PPRE. This site is not conserved in rats resulting in the lack of binding of PPAR to the rat apoA-I promoter. In contrast, rat apoA-I transcription is repressed by Rev-erb α , whose expression is induced by fibrates and which binds to a RevRE site adjacent to the TATA box in the rat, but not in the human apoA-I promoter. These observations indicate that the expression of the transcription factor Rev-erb α may be under control not only by pharmacological, but also by dietary factors (such as fatty acids) as well as inflammatory cytokines (such as PG-J2 and LT-B4), which all bind to and activate PPARs (2, 3, 76–78). Altogether these observations suggest a role for the nuclear orphan receptor Rev-erb α in metabolic control.

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Subchronic Toxicity of Fish Oil Concentrates in Male and Female Rats

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Summary There are an overwhelming number of reports indicating the beneficial effects of fish oil supplements in human and animal nutrition. The purpose of this study, second in a series, was to evaluate the effects, particularly those that may be harmful, of high-dose, long-term consumption of fish oil concentrates (FOC) using male and female rats. One hundred and twenty male and 120 female rats were gavaged daily with oils and oil mixtures in a volume equal to 0.5% body weight (5 mL/kg/d) for 13 weeks. The administered oils were corn oil, pure menhaden oil (MO), pure MaxEPA® fish oil or different mixtures of corn oil with MO. The stability and the homogeneity of the dosing solutions were tested under study conditions. The animals received isocaloric and isonitrogenous diets throughout. Food and pure water were supplied ad libitum. At the end of the in-life phase of the study, the animals were anaesthetized with CO₂ and humanely killed by exsanguination. Blood and other tissues were prepared for various clinical, histopathological and laboratory tests. Some beneficial effects of FOC, such as reduction in total serum cholesterol, in rats were confirmed. However, we also observed a significant reduction in absolute amount of serum HDL and a significant increase in relative liver and spleen weights in both sexes with the high dose of FOC. High doses of FOC (5 mL/kg/d) reduced serum iron and vitamin E concentrations. A reduction in osmotic fragility of RBC as well as an increase in RBC deformity were also observed in rats treated with high doses of FOC. These rats showed a significant overall increase in WBC count. We conclude that in rats, subchronic consumption of high levels of FOC can be beneficial but may also be harmful because of induction of clinical abnormalities including increased red cell deformity, increased relative liver and spleen weights, and reduced serum HDL, iron and vitamin E concentrations.

Key Words subchronic toxicity, fish oil concentrates, *n*-3 fatty acids

Fish oil concentrates (FOC) which contain omega-3 fatty acids are becoming increasingly popular due to their purported beneficial effects in prevention and/or treatment of many diseases including cardiovascular disease and cancer (1–5). Some scientists suggest prophylactic use of *n*-3 fatty acids through a broad food enrichment (6). Reports concerning the beneficial effects of FOC supplements in health and diseases are conflicting (7–10). Some reports indicate that there may be harmful effects associated with either prolonged ingestion or acute ingestion of large quantities of the omega-3 fatty acids (11). These include development of aneurisms and increased risk of stroke (12) and infections (13), modulation of the immune system (14), enhancement of ethanol- (15) or acetaminophen-induced hepatotoxicity (16), lipidoses (17), enhancement of fatty streak (18) and plaque formation (19) in the aorta, and detrimental effects on glucose tolerance in type II diabetics (20). Other harmful effects related to the consumption of FOC were reported in both animals (21) and humans (22). A study was conducted with MaxEPA®, a commercially available FOC, to obtain an indication of any clinical, biochemical, or pathological problems that might result from a 65-day (5 d/wk for

13 weeks) high-level administration of this FOC (23). This follow-up 90-day study (7 d/wk for 13 weeks) presented herein was performed using MO, a relatively crude fish oil that was well characterized and provided by the National Marine Fisheries Service (NMFS) for use in clinical or non-clinical studies. To facilitate comparisons with our previous study, twenty male and twenty female rats were also treated with MaxEPA® at the high dose level of 5 mL/kg/d for 13 weeks.

MATERIALS AND METHODS

The oils, animal care and treatments. The National Marine Fisheries Service (NMFS), Southeast Fisheries Center, Charleston Laboratory, Charleston, SC, USA, provided MO in polyethylene plastic containers. This crude fish oil contained a total of 29.9% *n*-3 fatty acids (FA) including 9.2% docosahexaenoic acid (DHA) and 12.9% eicosapentaenoic acid (EPA). Contaminants in the oil, determined by the NMFS, were polychlorinated biphenyl (PCB) <0.05 ppm and dichlorodiphenyl-trichloroethane (DDT) <0.004 ppm. The oil also contained 0.02% TBHQ, an antioxidant, and 2.2 mg/mL of vitamin E. MaxEPA® was provided by R.P. Scherer Company, Clearwater, FL, USA. This FOC provided a

total of 35% omega-3 FA, including 12% DHA and 17.8% EPA, and 2 mg vitamin E/g oil. Contaminants in the oil were determined by the manufacturer, and the values for PCB, Hg, Pb, As, and Cd were <1 ppm, and for pesticides, <0.1 ppm. Mazzola® Corn Oil (CO), the control article, was a gift from Dr. Mark Bieber (Best Foods, Union, NJ, USA). Samples of the CO were shipped to NMFS for the analysis of TBHQ and vitamin E. The oil was then fortified with vitamin E and TBHQ (Tenox 20A) to contain 0.02% TBHQ and 2.2 mg vitamin E per g of oil, an amount of these chemicals equal to that found in MO. The oils had a specific gravity of 0.93 g/mL. All the oils were diffused and blanketed with N₂ in the original plastic containers, protected against light and stored under similar conditions (−20°C) until used.

Four week-old male and female Sprague Dawley rats, 120 of each sex, were purchased from Charles River Breeding Lab. Inc., Wilmington, MA, USA (Raleigh, N.C. colony). The animals were quarantined and acclimated in the same room for 13 days. They were then weighed and grouped in 5 g intervals, randomized and assigned to six treatment groups of 20 rats per treatment per sex. The animals were eartagged for identification. The rats were housed, individually, in suspended stainless steel cages with screen bottoms. The animal room had a 12 h light-dark cycle, temperature of 18–24°C and a relative humidity of 40–70%. There was a morning and an afternoon check for sick or dead animals. Food and water were checked daily. During the weekly weighing and daily gavaging, the animals were thoroughly examined, and any signs of toxicity were recorded. Purina™ Ground Chow was supplied by Purina™ Mills Co., St. Louis, MO, USA, and stored at 4°C. Food and water (AQUA COOL® Ultrapure Water, Watertown, MA, USA) were supplied ad libitum during the quarantine and during the live-phase of the study. The feeding cups were filled with fresh food once a week, using a 1741 Dry Weight Dispenser (Volland Corp., White Plains, NY, USA), and weekly food con-

sumption was calculated. The diets were isocaloric and isonitrogenous throughout. The animal care was monitored by the FDA's Institutional Animal Care and Use Committee (IACUC). The *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources Council, National Academy of Sciences, 1986) was followed throughout. All clinical and laboratory tests adhered to the Good Laboratory Practice Guidelines.

The dosing solutions were administered daily for 13 weeks by gavage of oils in a volume equal to 0.5% of an animal's body weight. This procedure was previously described (23, 24). This amount of administered oil was high but did not cause diarrhea in the animals. The calculated human equivalent of the highest dose is about 19 g of *n*-3 FA/d for MO and 23 g of *n*-3 FA/d for MaxEPA® for a 65 kg human. Five mL of dosing solutions contained the following mL of MO diluted with corn oil: 0.0 (Control, pure corn oil); 0.1, 0.5, 2.5 and 5 (the highest dose, pure MO), representing treatment numbers I, II, III, IV and V, respectively (Table 1). Treatment number VI was pure MaxEPA®. Light-initiated autoxidation (25), was avoided by preparing the dosing solutions, 4 L at a time, under yellow light. The oils were then sampled from top, middle and bottom and tested for FA profile (26) to ensure homogeneity of the dosing solutions. A gas-liquid chromatograph (HP 5880A, Rockville, MD, USA) equipped with a wall coated open tubular capillary column (Supelco-10, 30 m, 0.32 mm id, and 0.25 µm df., Supelco, Supelco Park, Bellefonte, PA 16823) was used. The oils were then dispensed into 150 mL prescription bottles covered with aluminum foil, diffused and blanketed with N₂, capped, labeled and stored at −20°C until used. Each bottle was used for only one day of dosing. The stability data of all the oils, CO, MO, MaxEPA® and the oil content of the diet were provided by the suppliers. However, we also confirmed the data by measuring the FA profile of the oils and of the dosing solutions as well as their peroxide values (27). FA profile of the diet was also de-

Table 1. Description of the treatments and characteristics of the oils.

Treatment number ²	mL of		<i>n</i> -3/ <i>n</i> -6 Ratio	Concentrations of <i>n</i> -3 FAs (mg/g of oil) ¹		PV (meq/kg)
	Corn oil	Menhaden oil		EPA	DHA	
I	5.00	0.00	0.02	ND	ND	1.0±0.1
II	4.90	0.10	0.17	2.6±0.5	1.8±0.2	1.1±0.2
III	4.50	0.50	0.77	11.8±0.7	8.4±0.6	1.3±0.1
IV	2.50	2.50	3.79	58.8±0.7	42.7±0.6	2.1±0.1
V	0.00	5.00	7.55	116.9±2.3	85.6±1.6	2.8±0.2
		MaxEPA®				
VI	0.00	5.00	8.30	135.9±5.7	99.9±5.4	1.2±0.3

¹ The values are M±SE of seven duplicate determinations over 3 mo.

² Each oil treatment was administered daily in a volume equal to 0.5% body weight for 13 weeks.

Concentration of vitamin E in the dosing solutions (mg/g oil): treatment I (corn oil control), 1.00; II, 1.02; III, 1.12; IV, 1.35; V (pure Menhaden oil), 2.20; and VI (pure MaxEPA®), 2.00.

FA, fatty acids. EPA, eicosapentaenoic acids (20 : 5, *n*-3 FA). DHA, docosahexaenoic acid (22 : 6, *n*-3 FA). ND, not detected. PV, peroxide value.

Table 2. Stability tests: Fatty acid profiles of oils and extracted oil from food, and peroxide values at the beginning and at the end of the study.¹

Fatty acid	Beginning of the study				End of the study			
	CO	MO	Max	Chow	CO	MO	Max	Chow
C14:0	0.0	6.3	6.5	0.7	0.0	6.4	6.4	0.7
C14:1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1
C16:0	9.5	15.8	15.8	13.7	9.1	15.7	16.0	14.9
C16:1	0.1	8.4	7.9	0.9	0.1	8.4	7.7	1.0
C18:0	1.8	2.4	3.0	3.5	1.8	2.4	2.9	3.5
C18:1	29.3	10.8	8.0	19.8	29.3	10.4	7.4	21.1
C18:2	56.9	1.2	1.3	45.8	57.1	1.2	1.0	47.7
C18:3	0.9	1.0	0.8	4.2	0.9	0.9	0.7	4.7
C20:0	0.5	0.0	0.2	0.4	0.5	0.1	0.3	0.4
C20:1	0.2	2.9	1.5	0.5	0.3	2.9	1.7	0.5
C20:2	0.0	0.2	0.2	<0.1	0.0	0.2	0.1	<0.1
C20:3	0.0	0.2	0.1	<0.1	0.0	0.1	0.1	<0.1
C20:4	0.0	0.4	0.9	0.1	0.0	0.4	0.9	0.1
C20:5	0.0	15.0	17.1	1.2	0.0	15.1	17.9	1.3
C22:0	0.1	0.1	0.1	0.4	0.1	0.1	0.1	0.4
C22:1	0.0	0.3	0.2	0.2	0.0	0.3	0.1	<0.1
C22:6	0.0	11.2	12.8	<0.1	0.0	11.2	13.6	<0.1
C24:0	0.1	0.0	0.0	1.4	0.2	0.0	0.0	1.5
C24:1	0.0	0.5	0.7	<0.1	0.0	0.5	0.6	<0.1
Others	0.6	23.2	22.9	7.0	0.6	23.6	22.5	2.0
PV (meq/kg)	0.8	2.7	0.8	ND	1.0	2.8	1.4	ND

¹ The values for fatty acid profiles are the average of three determinations and are expressed as % of total oil.

CO, corn oil control. MO, menhaden oil. Max, MaxEPA® oil. Chow, Ralston Purina™ Ground Chow (No. 5002). PV, peroxide value. ND, not determined.

terminated at the beginning and at the end of feeding period (Centech Laboratories St. Louis, MO, USA) to provide stability data for the test and control articles and the data are summarized in Table 2.

Euthanasia, necropsy and clinical tests. At the conclusion of the in-life phase of the study, animals were anaesthetized with excess CO₂, weighed, humanely killed by exsanguination via the posterior vena cava, and necropsied. During each day of necropsy, four rats from each treatment group (total of 24 rats from each sex) were randomly selected for euthanasia. Blood was drawn using a 20 mL plastic syringe and a stainless steel needle, 21 g and 1.5 inch long. Osmotic fragility of the red blood cells (OFRBC) was determined immediately according to the method of Dacie and Lewis (28). Prothrombin time (PT) and activated partial thromboplastin time (aPTT), two of the many coagulation indices, were determined by using Coag-A-Mate Model X2 (Organon Teknika, Oklahoma City, OK, USA), immediately after necropsy. Whole blood, serum and plasma specimens were also prepared for hematological and clinical chemistry tests by MetPath company (MetPath Inc., Rockville, MD, USA). These included complete blood count (CBC) with differentials, platelet counts, hemoglobin and hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), red blood cell distribution width (RDW), mean platelet volume (MPV), % neutrophils, lymphocytes, monocytes, eosinophil and platelets, glu-

cose, blood urea nitrogen (BUN), creatinine, BUN/creatinine ratio, Na, K, Cl, Ca, P, Fe, uric acid, total serum protein, albumin (A), globulin (G), A/G ratio, serum glutamate oxaloacetate transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), SGOT/SGPT ratio, γ -glutamyl-transpeptidase (GGTP), alkaline phosphatase, lactic dehydrogenase (LDH), total and direct bilirubin, triglycerides, cholesterol, high density lipoprotein (HDL) and serum vitamin E.

A thorough post-mortem gross examination was performed, and it included all external orifices, the pelvic, abdominal, thoracic and cranial cavities with their viscera and the carcass. Special attention was given to the aorta and distal portion of the colon for possible gross lesions. The following organs were removed, cleaned and weighed: adrenal glands, brain, heart, kidneys, liver, spleen, testes, ovaries and thymus. A full screen histopathology evaluation was performed on the following tissues/organs: adrenal glands, aorta, brain, sternum, esophagus, epididymis, one eye, heart, kidneys, lung, liver, pituitary gland, mammary glands, ovary, pancreas, prostate, submaxillary salivary glands, seminal vesicles, skeletal muscle (psoas), skin (right thoracic), stomach (glandular and non-glandular), spleen, testes, thymus, thyroid, parathyroid, trachea, urinary bladder and uterus. All trimmed tissues were processed, embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin and examined by light microscopy. Special stains, oil-red-o and osmium tetroxide

for fat, periodic acid schiff (PAS) reaction with and without diastase for glycogen, Gomori for iron and alizarin for calcium were used on selected sections of lung, liver, kidneys and spleen. Bone specimens (sternum) were removed, cleaned and decalcified in a solution composed of equal volume of 45% formic acid and 25% of sodium citrate before being processed as for other tissues. Likewise, duodenum, cecum, colon, and rectum were prepared and processed as described.

Liver specimens were collected for a qualitative histochemical test for peroxisomes (29). This test was performed on liver specimens, 3 rats for each of the six treatment groups per sex. The liver specimens were coated with starch powder, frozen in liquid nitrogen, placed into precooled vials and stored at -30°C until further processing. Ten micron sections of the tissues were obtained using a cryostat (-15°C) and were then prefixed with a 10% cold formalin (pH=7) for 15 minutes and incubated in filtered diaminobenzidine solution (DAB) for 1 h. The medium consisted of 10 mL of 1% H_2O_2 , 49 mL of 50 mM 2-amino-2-methyl-1,3-propanediol buffer (pH=8.8), 100 mg of DAB and 5 mL of 1 mM MnCl_2 . The sections were then counter stained with Mayer's hematoxylin, washed with tap water, dehydrated with alcohol and xylene and mounted. The peroxisomes were identified as brownish black areas under a microscope.

Statistical Methods. Mean separation using one and two-way analysis of variance (ANOVA) or covariance with appropriate comparisons in the presence of significant ANOVA was used to determine dose, sex and dose $\times$ sex interactions (30). Sex effects were determined by simple *t*-test. Dose effects were determined by mean comparisons using a two-sided LSD test. In the absence of significant dose and sex interactions, the overall effects were tested by two-way ANOVA. The Shapiro-Wilk test for normality of values for all the variables was used. When the test indicated that the values were not "normal", nonparametric procedures were used for statistical analysis of that variable. All differences with

$p \leq 0.05$ were deemed statistically significant.

RESULTS

Average weekly food consumption and weekly weight gain are summarized in Table 3. Fish oil consumption showed little or no effect on these parameters except at the highest dose level where food consumption increased significantly. In general, the average weekly weight gain was not affected by treatment. High doses of fish oils caused a significant increase in relative liver and spleen weights (Table 4), but did not affect the relative weights of other tissues.

Serum triglyceride (TG) level was not affected by fish oil, while the total serum cholesterol (TCh) and absolute amount of serum HDL were reduced significantly. These effects were more pronounced in female than in male rats (Table 5). However, both total cholesterol and the HDL data in the three female groups (control, treatments I & II) had very high standard deviations. Nevertheless, the dose effect toward lower serum TCh and HDL determined by a two-way ANOVA, was highly significant ($p < 0.0002$).

Two of the factors involved in the coagulation process, plasma PT and aPPT, were not affected by fish oil treatments (Table 6). A 10% increase in the white blood cell count was observed in both sexes. This increase in leukocyte count was statistically significant when the male and female data were pooled and a two-way ANOVA was applied ($p < 0.0056$). The mean platelet volume in the two highest dose groups was significantly reduced while the RDW was significantly elevated in both sexes in the highest dose levels. There was also a trend in lessening of OFRBC, but this was statistically significant only when two-way ANOVA was applied (Table 6). Clinical chemistry data are presented in Table 7. Serum concentration of BUN increased in female rats receiving the highest dose of MO, while the concentration of serum creatinine significantly declined in the female rats. In male rats, some serum enzyme activities (SGOT and LDH) were affected by the high doses

Table 3. Average weekly food consumption and weight gain.

Treatment ¹ (mL of FO/kg/d)	g/wk/rat			
	Food consumption		Weight gain	
	Male	Female	Male	Female
0.00 (corn oil control)	138 $\pm$ 14	105 $\pm$ 9	26 $\pm$ 4	10 $\pm$ 3
0.10	145 $\pm$ 9	106 $\pm$ 11	28 $\pm$ 3	10 $\pm$ 2
0.50	140 $\pm$ 16	105 $\pm$ 12	25 $\pm$ 5	10 $\pm$ 2
2.50	146 $\pm$ 17	109 $\pm$ 12	27 $\pm$ 5	11 $\pm$ 2
5.00 (menhaden oil) ²	147 $\pm$ 11 ^a	112 $\pm$ 9 ^c	26 $\pm$ 4	12 $\pm$ 2 ^e
5.00 (MaxEPA [®] oil) ²	151 $\pm$ 16 ^b	114 $\pm$ 7 ^d	27 $\pm$ 4	11 $\pm$ 2
Dose effect ³	$p < 0.0005$		NS	

¹ The oils were administered daily in a volume equal to 0.5% body weight for 13 weeks.

² Frequent food spillage in high dose groups.

³ By two-way ANOVA (no significant dose $\times$ sex interaction).

NS, statistically not significant; ^a $p < 0.0298$, ^b $p < 0.0034$, ^c $p < 0.0238$, ^d $p < 0.0069$, ^e $p < 0.0057$.

The values are $M \pm SD$ of 19–20 rats. FO, fish oil.

Table 4. Relative liver and spleen weights.

Treatment ¹ (mL of FO/kg/d)	Liver (g/100 g)		Spleen (mg/100 g)	
	Male	Female	Male	Female
0.00 (corn oil control)	3.6±0.55	3.7±0.39	158±31	189±23
0.10	3.5±0.25	3.7±0.54	151±28	192±21
0.50	3.5±0.22	3.8±0.52	160±24	185±39
2.50	3.6±0.24	4.0±0.57	154±26	197±46
5.00 (menhaden oil)	3.9±0.34 ^a	4.0±0.37 ^c	171±22	205±33 ^f
5.00 (MaxEPA [®] oil)	4.0±0.39 ^b	4.0±0.38 ^d	175±25 ^e	203±23
Dose effect ²	$p<0.0001$		$p<0.0110$	

¹ The oils were administered daily in a volume equal to 0.5 % body weight for 13 weeks.

² 2-Way ANOVA (no significant dose×sex interaction).

FO, fish oil; ^a $p<0.0006$, ^b $p<0.0010$, ^c $p<0.0246$, ^d $p<0.0326$, ^e $p<0.0035$, ^f $p<0.0271$.

The values are M±SD of 19–20 rats.

Table 5. Concentrations of serum triglycerides, total cholesterol and high density lipoprotein.

Treatment ¹ (mL of FO/kg/d)	TG (mg/100 mL)		TCh (mg/100 mL)		HDL (mg/100 mL)	
	Male	Female	Male	Female	Male	Female
0.00 (corn oil control)	85±36	86±40	49±9	59±15	40±9	54±14
0.10	95±45	83±43	50±9	57±12	41±8	54±10
0.50	102±43	74±37	51±7	57±16	41±7	55±12
2.50	89±47	78±35	45±6	51±9	36±6	50±12
5.00 (menhaden oil)	89±31	62±23	47±11	44±10 ^b	34±10 ^d	42±12 ^f
5.00 (MaxEPA [®] oil)	86±41	64±24	43±6 ^a	46±9 ^c	32±7 ^e	44±10 ^g
Dose effect ²	NS		$p<0.0002$		$p<0.0002$	

¹ The oils were administered daily in a volume equal to 0.5 % body weight for 13 weeks.

² By two-way ANOVA.

NS, statistically not significant; ^a $p<0.0311$, ^b $p<0.0028$, ^c $p<0.0152$, ^d $p<0.0245$, ^e $p<0.0026$, ^f $p<0.0002$, ^g $p<0.0003$.

The values are M±SD of 19–20 rats. FO, fish oil. TG, triglycerides. TCh, total cholesterol. HDL, high density lipoprotein.

of fish oil intake. While serum chloride concentration was slightly elevated in the high-dose male rats, serum phosphorus level was slightly reduced in the highest dose of MO treated female rats (Table 8). The dose effect, as determined by two-way ANOVA, was statistically significant. The data on serum levels of iron and vitamin E show a significant decrease primarily in female rats (Table 8). To examine the overall dose response of the variables mentioned in this study, a linear correlation between dose levels and the variables was performed, and the data are summarized in Table 9. The following parameters were similarly affected in both sexes: relative liver weight, RDW, MPV, OFRBC, serum phosphorous, vitamin E and HDL. The histochemical analysis of the liver peroxisomes (CAT enzyme) showed no sign of peroxisomal proliferation (data not shown).

DISCUSSION

Our study of FOC was designed with dosing solutions with parameters similar to those used by other investigators (31) who based their studies on the *n*-3/*n*-6 ratios (Table 1). These values were similar to the fish oil values in the dosing solutions, and we used the actual amount of fish oil for the statistical analysis rather than

the ratios of the fish oil components. During the course of the study, the EPA and DHA concentrations of the dosing solutions as well as the FA profile of the oils were monitored along with their peroxide values (PV) to ensure the use of homogeneous and stable doses throughout.

It is known that rats treated with fish oil tend to spill food (32). In our study this was observed in the high-dose groups (Table 3). Although food consumption was significantly higher in the high-dose groups, little or no difference was observed in average weekly weight gain, the female rats on high-doses of MO excepted. These animals gained about 2 g/wk more than the control group. Although this small weight gain was statistically significant ($p<0.0057$), it seems unlikely that this factor affected the outcome of this study. In fact, the two-way ANOVA showed no significant dose effect on weight gain despite a highly significant effect on apparent food consumption (Table 3, $p<0.0005$). The data presented in Tables 4 to 9, cannot be explained by factors other than our treatment.

Similar to the results of our earlier study with MaxEPA[®], the relative liver and spleen weights were significantly elevated with the highest doses of FOC (Table

Table 6. Some hematological data.

Treatment ¹ (mL of FO/kg/d)	Male	Female	Male	Female	Male	Female
	PT (s)		aPTT (s)		OFRBC (%)	
0.00 (corn oil control)	11±0.4	10±0.4	17±5	16±3	72±11	83±11
0.10	10±0.6	10±0.7	16±1	17±3	76±13	82±14
0.50	11±0.3	10±0.8	16±2	18±3	73±12	84±9
2.50	11±0.6	11±0.7	18±7	18±3	69±15	82±10
5.00 (menhaden oil)	11±0.8	10±0.6	15±2	17±2	67±14	74±15
5.00 (MaxEPA® oil)	11±0.4	10±0.5	15±2	17±2	69±16	79±10
Dose effect ²	NS		NS		p<0.0479	
	WBC (10 ³ /mm ³)		MPV (fL)		RDW (%)	
0.00 (corn oil control)	13.4±3	9.4±2	8.5±0.6	8.2±0.6	14±0.6	12±0.6
0.10	12.5±4	9.3±2	8.4±1.4	8.0±0.7	14±0.8	13±0.9
0.50	12.5±3	9.4±3	8.2±0.6	8.1±0.6	15±0.7	13±0.8
2.50	14.7±4	10.0±3	7.7±0.7 ^a	7.4±0.9 ^d	15±0.5	13±0.9
5.00 (menhaden oil)	15.1±2	10.4±2	7.6±0.6 ^b	7.2±0.4 ^e	15±1.3 ^f	13±0.6 ^h
5.00 (MaxEPA® oil)	14.7±3	10.9±2	7.9±0.7 ^c	7.1±0.6 ^e	15±0.8 ^g	13±1.5 ⁱ
Dose effect ²	p<0.0056		p<0.0002		p<0.0002	

¹ The oils were administered daily in a volume equal to 0.5% body weight for 13 weeks.

² By two-way ANOVA.

NS, statistically not significant; ^a p<0.0044, ^b p<0.0029, ^c p<0.0469, ^d p<0.0009, ^e p<0.0002, ^f p<0.0014, ^g p<0.0379, ^h p<0.0004, ⁱ p<0.0003.

The values are M±SD of 17–20 rats. FO, fish oil. PT, prothrombin time. aPTT, activated partial thromboplastin time. OFRBC, osmotic fragility of red blood cells. WBC, white blood cell count. MPV, mean platelet volume. RDW, red blood cell distribution width.

Table 7. Clinical chemistry data.

Treatment ¹ (mL of FO/kg/d)	Male	Female	Male	Female	Male	Female
	BUN (mg/dL)		Creat. (μg/dL)		BUN/Creat. Ratio	
0.00 (corn oil control)	13.8±2	15.4±2	680±130	675±80	20.8±4	23.1±4
0.10	14.0±2	16.2±2	639±80	635±110	22.4±5	26.8±9
0.50	14.0±2	16.7±3	635±90	615±170	22.6±5	30.0±13
2.50	13.6±2	17.1±3	605±180	590±190 ^b	24.3±8	32.8±16 ^e
5.00 (menhaden oil)	14.9±2	18.2±4 ^a	620±160	616±80 ^c	25.7±9	30.1±10 ^f
5.00 (MaxEPA® oil)	14.4±2	16.1±2	645±110	585±60 ^d	22.7±5	27.7±5 ^g
Dose effect ²	p<0.0222		NS		p<0.0093	
	SGOT (IU/L)		SGOT/SGPT Ratio		Total LDH (U/L)	
0.00 (corn oil control)	122±35	143±63	1.9±0.4	2.0±0.7	756±312	588±339
0.10	133±48	143±82	1.6±0.5 ⁱ	2.1±0.6	862±599	595±409
0.50	130±42	109±34	1.5±0.4 ^j	2.0±0.5	950±674	389±168
2.50	104±34 ^h	123±55	1.5±0.3 ^k	2.0±0.5	560±404 ⁿ	491±309
5.00 (menhaden oil)	113±30	113±55	1.3±0.3 ^l	1.8±0.3	564±421 ^o	407±251
5.00 (MaxEPA® oil)	127±46	130±51	1.6±0.3 ^m	2.0±0.5	673±542	610±400
Dose effect ²	p<0.0117		NS		NS	

¹ The oils were administered daily in a volume equal to 0.5% body weight for 13 weeks. FO, fish oil.

² By two-way ANOVA.

NS, statistically not significant; ^a p<0.0028, ^b p<0.0351, ^c p<0.0452, ^d p<0.0390, ^e p<0.0018, ^f p<0.0006, ^g p<0.0093, ^h p<0.0229, ⁱ p<0.0126, ^j p<0.0039, ^k p<0.0009, ^l p<0.0002, ^m p<0.0096, ⁿ p<0.0390, ^o p<0.0081.

The values are M±SD of 17–20 rats. BUN, blood urea nitrogen. Creat., serum creatinine. SGOT, serum glutamic oxaloacetate transaminase. SGPT, serum glutamic pyruvic transaminase. LDH, serum lactic dehydrogenase.

Table 8. Serum levels of some minerals and vitamin E.

Treatment ¹ (mL of FO/kg/d)	Male	Female	Male	Female
	Chloride (mm/L)		Phosphorous (mg/100 mL)	
0.00 (corn oil control)	94.7±2.0	95.3±2.7	11.5±1.3	9.5±1.1
0.10	94.5±3.1	96.5±3.0	11.0±1.3	9.3±0.9
0.50	95.8±2.6	97.5±2.6	11.5±1.6	8.9±1.0
2.50	95.4±2.3	96.9±2.8	11.0±1.1	9.4±1.4
5.00 (menhaden oil)	96.4±2.6 ^a	97.6±2.7	10.6±1.4	8.6±0.8 ^c
5.00 (MaxEPA [®] oil)	97.0±2.6 ^b	97.0±3.5	10.7±1.1	9.1±1.1
Dose effect ²	$p<0.0028$		$p<0.0216$	
	Iron (µg/100 mL)		Vitamin E (mg/L)	
0.00 (corn oil control)	247±37	475±95	13.7±3.2	20.4±5.4
0.10	238±28	461±106	15.2±5.5	20.8±5.8
0.50	236±25	473±99	4.5±3.5	20.3±6.8
2.50	247±37	365±80 ^d	12.9±3.8	16.3±6.9 ^h
5.00 (menhaden oil)	240±41	363±92 ^d	9.7±2.7 ^f	10.5±3.5 ⁱ
5.00 (MaxEPA [®] oil)	231±36	388±55 ^e	11.4±3.2 ^g	12.5±3.1 ⁱ
Dose effect ²	$p<0.0002$		$p<0.0002$	

¹ The oils were administered daily in a volume equal to 0.5 % body weight for 13 weeks.

² By two-way ANOVA.

FO, fish oil; ^a $p<0.0281$, ^b $p<0.0035$, ^c $p<0.0109$, ^d $p<0.0003$, ^e $p<0.0029$, ^f $p<0.0012$, ^g $p<0.0512$, ^h $p<0.0183$, ⁱ $p<0.0002$.

The values are M±SD of 18–20 rats.

Table 9. Regression of some variables on the gavaged dose levels.

Variables	Male		Female	
	$r^1=$	$p<$	$r^1=$	$p<$
Liver (g/100 g Bwt.) ²	0.311	0.0017	0.375	0.0002
Spleen (mg/100 g Bwt.)	NS	NS	0.342	0.0006
Gonads (mg/100 g Bwt.)	NS	NS	0.340	0.0007
BUN (mg/100 mL)	NS	NS	0.280	0.0051
BUN/Creatinine ratio	0.254	0.0123	NS	NS
Uric acid (mg/100 mL)	NS	NS	-0.240	0.0169
MCHC (%)	0.224	0.0320	NS	NS
WBC count (10 ³ /mm ³)	0.286	0.0058	NS	NS
RDW (%)	0.382	0.0003	0.332	0.0013
MPV (fL)	-0.362	0.0005	-0.478	0.0002
OFRC (%)	-0.196	0.0506	-0.253	0.0117
APase (IU/L) ³	0.221	0.0297	NS	NS
LDH (U/L) ³	-0.245	0.0156	NS	NS
SGOT/SGPT ratio	-0.365	0.0003	NS	NS
Chloride (mm/L) ³	0.228	0.0250	NS	NS
Calcium (mg/100 mL) ³	0.292	0.0039	NS	NS
Phosphorous (mg/100 mL) ³	-0.215	0.0344	-0.203	0.0441
Iron (µg/100 mL) ³	NS	NS	-0.444	0.0002
Vitamin E (µg/100 mL) ³	-0.433	0.0002	-0.565	0.0002
Cholesterol (mg/100 mL) ³	NS	NS	-0.342	0.0006
HDL (mg/100 mL) ³	-0.327	0.0012	-0.437	0.0002
Triglycerides (mg/100 mL) ³	NS	NS	-0.200	0.0474

¹ Correlation coefficient.

² Bwt., body weight.

³ In serum; BUN, blood urea nitrogen. MCHC, mean cell hemoglobin concentration. WBC, white blood cell count. RDW, red blood cell distribution width. MPV, mean platelet volume. OFRC, osmotic fragility of red blood cells. APase, alkaline phosphatase. LDH, lactic dehydrogenase. SGOT, serum glutamic oxaloacetate transaminase. SGPT, serum glutamic pyruvic transaminase. HDL, high density lipoprotein.

4). In the human population, hepatosplenomegaly has been reported as a result of nutritional zinc deficiency (33). Although we did not assess the mineral status of animals, it is possible that a mild zinc deficiency linked to decreased mineral absorption was caused by high fish oil intake. Enlargement of the liver and spleen in our study did not appear to be as severe as that observed in human zinc deficiency. In any event, the pathological examinations of these organs failed to provide adequate morphological and/or other information to explain the increase in relative organ weights. An accurate test of peroxisomal proliferation would have been useful in explaining the increase in relative organ weight (34). However, in a recent report on mice treated with fish oil for 30 days, a 40% increase in spleen size was observed when compared to the soybean oil control group (35). This confirms our finding on enlargement of spleen size in rats as a result of treatment with Menhaden oil (Table 4). A significant increase in the concentration of RBC phosphatidylcholine hydroperoxide as well as phosphatidyl-ethanolamine hydroperoxide was also found in the mice treated with fish oil (35). The histopathological data on most of the tissues we examined were unremarkable.

In the present study, a 23 to 26% reduction in serum TG was observed in the high-dose female groups. Statistically, this reduction was not significant (Table 5). A study of 19 healthy human volunteers who received 6 g/d of MaxEPA fish oil for 21 days also showed no significant change in serum TG (36). In contrast, other investigators have reported a significant reduction in serum TG in miniature pigs treated with fish oil (37), in hypertriglyceridemic subjects receiving PUFA (38) and in female rats treated with MaxEPA in an amount equal to 0.5% body weight, 5 days a week for 13 wk (23). Moreover, a recent review on the subject (39) revealed a dose response decrease in blood TG levels in hypertriglyceridemic patients receiving high doses of fish oil supplements which persisted as long as supplementation continued. Although the values for female rats were within the reported normal range (40), the negative correlations between dose levels and serum TG were significant in this study (Table 9) and in our previous study (23) which confirms the TG-lowering effect of fish oil in female rats. The total cholesterol values were within the reported normal range (40) but were significantly reduced (22%) by treatment with the high doses of fish oil (Table 5). These data support the findings by others who found significant reduction in serum cholesterol caused by fish oil ingestion (41), possibly through increased cholesterol secretion into bile (42). However, this was associated with a significant reduction (22%) in absolute amount of serum HDL level as well (Table 5). The two-way ANOVA indicates highly significant dose effect in reducing serum cholesterol and HDL ($p < 0.0002$). In fact, the HDL-lowering effect of dietary fish oil or *n*-3 fatty acids has been reported in rats (43), hamsters (44), pigs (45) and monkeys (46). However, review of literature revealed that in humans fish oil consumption may actually increase serum HDL

level (47, 48).

Fish oil consumption is known to increase bleeding time in rabbits (49) and in humans (12). While we did not directly measure this variable, our results of PT and aPTT (Table 6), two factors involved in blood clotting, indicated no treatment effect. These results agree with those obtained from a study with human subjects who received 6 g DHA/d for 90 days (50) and confirm our previous findings (23) that fish oil consumption in rats had no effect on these parameters. The OFRBC in females however, was reduced by 9%. We can not explain this sex difference in the susceptibility to changes in OFRBC after fish oil treatment. Nevertheless, there was a weak, but significant, correlation between dose levels and OFRBC in both sexes as indicated in Table 9. A possible explanation for this finding is provided by the reports that the ingested *n*-3 FA can be incorporated into the cell membrane (51) causing higher membrane fluidity, greater flexibility of the cell membrane and increased resistance to osmotic pressure. When rats were fed a diet containing 10% salmon oil for 3 weeks, their erythrocytes became osmotically less resistant to NaCl solutions when compared to rats on a 10% coconut oil diet (52). Erythrocytes obtained from healthy human volunteers receiving 7.7 g of *n*-3 FA/d for 180 days showed, among other observations, a decrease in susceptibility to osmotic hemolysis (53). The authors concluded that high doses of *n*-3 FA can modify erythrocytes' structure and function with resultant harmful side effects.

High doses of FOC increased the leukocyte count from 10% (male rats, MaxEPA®) to 16% (female rats, MaxEPA®). Although this increase was statistically insignificant, the pooled data on both sexes showed a highly significant dose effect ($p < 0.0056$, Table 6). There was a small but highly significant correlation coefficient between the two variables in male rats only ($p < 0.0058$, Table 9). The elevated WBC count may be a response to high doses of FOC. However, values in the control and treated female rats were within the reported normal range of 6.6–12.6 but in male rats were beyond the reported normal range of $8\text{--}11.8 \times 10^3/\text{mm}^3$ of blood (54). Although the platelet count showed no treatment effect (data not shown), the MPV was significantly decreased even at the medium dose levels and in both sexes (Table 6). The pooled male and female data showed a highly significant dose effect on MPV ($p < 0.0002$). Our finding is in agreement with a recent report on rats (55) but contrary to the results obtained from a human study (56). This latter study showed that when 266 healthy volunteers received 15 mL/d of cod liver oil/olive oil or whale oil for 12 weeks, their MPV values were elevated by 8%. This discrepancy may be explained by different administered dose levels to two different species and for different duration.

Another structural change in blood cells of rats treated with high doses of fish oil was a significant increase in RDW in both sexes (Table 6). It may be speculated that this was a compensatory response to a subtle

hemolysis, as evidenced by enlarged spleen (Table 4), and/or to a diminished intestinal iron absorption caused by high level ingestion of PUFA. In fact, this may be supported by another finding, e.g. a decrease in serum iron, especially in the high-dose female rats (Tables 8 and 9). Increase in the RDW rate has been reported in infant anemia (57) and in α -thalassemia (58). However, we found no sign of anemia in the FOC treated animals, and we cannot explain with certainty the underlying mechanism and biological consequences of this observation on RDW.

In our study (Table 7), blood urea nitrogen (BUN) was elevated by 8% in male (statistically not significant) and 18% in female rats ($p < 0.0028$). Since in rats, BUN level does not appear to vary with sex or age (59), we cannot explain the sex difference in BUN response to high doses of FOC. In humans with acute renal failure, the BUN level can be used as an indicator of protein catabolism (60). Although the BUN values in our study were within the reported normal range (46), the pathology data did not show kidney and/or renal tubular damage, which may have accounted for the high level of BUN in female rats ($p < 0.0222$). However, as indicated in Table 4, the high-dose treated female rats exhibited an 8.5% increase in relative liver weight. This may partially explain the increase in their BUN level. Creatinine is a product of muscle metabolism, and its serum levels are directly related to muscular conditioning. According to Everett and Harrison (61), factors that increase serum creatinine levels will also elevate the BUN levels. The data presented in Table 7 contradict this conclusion by Everett and Harrison because the BUN level slightly increased in high-dose groups, while the serum creatinine levels significantly decreased ($p < 0.0390$). The animals on high doses of FOC were more sedentary, thus rendering lower serum creatinine levels. It is important to note that the BUN/Creatinine ratios were significantly increased in the high dose groups of female and that the overall dose effect on the ratio of these variables was highly significant (Table 7, $p < 0.0093$). In any event, all the BUN and creatinine values presented in Table 7 were within the normal range reported in the literature (54), and these changes may be considered biologically insignificant. There was a significant dose effect on the SGOT values ($p < 0.0117$). Also the SGOT/SGPT ratios were reduced at all dose levels. However, the SGOT values were beyond the average values reported in the literature (54) making it difficult to interpret the data. Likewise, the data on serum total LDH are directionally inconsistent.

The blood electrolyte data show that serum chloride values increased significantly (MO, $p < 0.0281$ and MaxEPA®, $p < 0.0035$, Table 8) with high doses of fish oil in male rats. However, these values were below those reported in the normal range (54). In our study, the overall dose effect in the pooled male and female data was highly significant ($p < 0.0028$). Wickwire et al. (62) reported accelerated renal disease in rats fed a 9% MO diet; Tateno et al. (63) reported a BSA-induced immune complex nephritis in mice fed a casein-based diet con-

taining 10% fish oil concentrate. Therefore, in conjunction with the observed increase in BUN/Creatinine ratio (Table 7), further investigation is warranted on the effects of long-term, high-dose intake of fish oil concentrates on renal function. As shown in Table 8, a modest reduction in serum phosphorous level was observed in the high-dose female rats ($p < 0.0109$). The pooled male and female data also showed a significant dose effect ($p < 0.0216$). However, our values seem to be within the upper limits of the reported normal range (54), and it probably should be considered a statistical aberration without biological significance.

Our data showed that FOC lowered serum iron level in female rats by 34% (Table 8). We are unable to explain the apparent susceptibility of serum iron in female rats to FOC, but this finding confirmed our previous study (23). However, in the present study, the serum iron values in both sexes were above the reported normal values (54). In humans, a daily intake of 20 mL of FOC in both sexes for ten weeks resulted in a significant reduction in serum and erythrocyte selenium concentrations (64). Rats receiving sardine oil for 4 weeks, showed a significant reduction in dietary iron absorption and retention (65). Another potentially harmful effect of high doses of FOC was a 48% reduction in serum vitamin E level in female rats and a 17% reduction in male rats both of which were statistically significant (Table 8). The overall dose effect on both parameters, serum iron and vitamin E, were highly significant ($p < 0.0002$). These findings are in agreement with the report of a significant decrease in both serum and liver concentration of vitamin E when rats were fed diets high in FOC (66). Likewise, our earlier studies showed that gavaging MaxEPA® to rats (23) or feeding MO to miniature swine (67) resulted in a significant reduction in vitamin E levels in serum and in liver, respectively.

High levels of dietary polyunsaturated fatty acids (PUFA) appears to reduce the intestinal absorption of iron (Table 8) while increasing the body's demand for vitamin E (68, 69). It must be noted that all the oils used in this study were adequately and equally fortified with both TBHQ and vitamin E. It is possible that the vitamin E intake was either inadequate and/or not absorbed in amounts sufficient to compensate for the additional burden imposed on the antioxidant system of the animals by the high intake of PUFA, leading to reduced body store of vitamin E.

The dose response of some variables are presented in Table 9. Negative control groups for both sexes to eliminate possible oil effect were not included in the study. Although some of the p values were highly significant, the overall correlation coefficients were small and the effects were apparent mostly at the highest dose levels. Nevertheless, some of these changes may be biologically significant. The positive correlations between dose levels and relative organ size and RDW may be considered unwanted side effects of consumption of high quantities of fish oil concentrates. Likewise, with the exceptions of OFRBC and serum phosphorous levels, all the negative correlations between dose levels and other parameters

were highly significant. That included serum iron in female rats ($r = -0.444$, $p < 0.0002$) and serum vitamin E and absolute amount of HDL concentrations in both sexes.

While long-term administration of high doses of fish oil had little or no effect on the average weekly weight gain of the rats, it caused a significant increase in relative liver and spleen weights. The mean platelet volume and osmotic fragility of red blood cells were reduced which indicates an effect on biological membrane structure and possibly on membrane functions. This study, as well as the previous study on MaxEPA®, appears to confirm some beneficial effects of fish oil consumption in the reduction of total serum cholesterol level in male and female rats. However, as a concomitant reduction in the serum HDL levels was seen as well, the benefit of this effect should be interpreted and extrapolated to humans with extreme caution. An important finding, not reported by others, was the adverse effect of fish oil on total serum iron in female rats. In confirmation of reports by others and by us, we also observed a significant reduction in serum vitamin E levels in male and female rats treated with the high doses of fish oils concentrates. We conclude that rats, especially females, given sub-chronic and high doses of FOC develop potentially harmful changes in several biochemical indices. These include increased red cell deformity, increased relative liver and spleen weights, and reduced MPV, absolute amount of serum HDL, serum iron and serum vitamin E concentrations.

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Docosahexaenoic Acid Increases Thyroid-Stimulating Hormone Concentration in Male and Adrenal Corticotrophic Hormone Concentration in Female Weanling Rats^{1,2}

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ABSTRACT Circulating levels of thyroid-stimulating hormone (TSH), growth hormone (GH), adrenal corticotrophic hormone (ACTH) and prolactin (PRL) were assessed in suckling rats in the postweaning period after rats were fed diets that reflect the fat composition of a current infant formula with or without the addition of 1.2 g/100 g fatty acid arachidonic acid [20:4(n-6)] and 0.7 g/100 g fatty acid docosahexaenoic acid [22:6(n-3)] or both 20:4(n-6) and 22:6(n-3). At 2 wk of age, no effect of diet on circulating levels of TSH, ACTH, GH or PRL was apparent. By 6 wk of age (3 wk postweaning), male rats consuming the diet containing 22:6(n-3) had significantly elevated levels of TSH, and females had significantly higher ACTH concentrations than males. No effect of diet was observed on circulating GH or PRL levels. Male pups had higher levels of TSH than females ($P < 0.0001$), whereas female pups from the 22:6(n-3) diet treatment exhibited much higher levels of ACTH than all male pups from any of the other diet treatments. These results suggest that metabolic controls, functioning through endocrine mechanisms, can be altered by changing the 20:4(n-6) to 22:6(n-3) balance in the diet. *J. Nutr.* 128: 1257–1261, 1998.

KEY WORDS: rats diet fat thyroid-stimulating hormone adrenal corticotrophic hormone growth

On the basis of the levels of essential fatty acids present in human milk and their accretion rate in the fetus during the last trimester of gestation, we concluded that infant formulas should contain the longer-chain homologues of 18:2(n-6) and 18:3(n-3) (Clandinin et al. 1982). It was our conviction that both 20:4(n-6) and 22:6(n-3) should be present in formulas in proportions that reflected the overall fatty acid balance found in the fatty acid mixture of human milk (Clandinin et al. 1989). We further suggested that supplementation of feedings with only very long-chain (n-3) fatty acids ($>18C$) and 18:2(n-6), although easily achieved, would not be as desirable because of predictable effects on the reduction in synthesis, metabolism and incorporation of 20:4(n-6) into membrane phospholipids. Feeding human milk results in increased content of 20:4(n-6) and 22:6(n-3) in erythrocyte phosphatidylethanolamine and phosphatidylcholine in term infants compared with similar membrane fractions for infants fed formula containing 18:2(n-6) and 18:3(n-3) but not their longer-chain homologues (Putnam et al. 1982). Moreover, when the formula has been modified to contain the longer-chain homologues of 18:2(n-6) and 18:3(n-3), the levels of 20:4(n-6) and 22:6(n-3) in plasma phospholipid and cholesterol ester are

similar to those found in infants fed human milk (Clandinin et al. 1992, Koletzko et al. 1987). In studies in which the feedings have been supplemented with very long-chain (n-3) fatty acids alone, there is a clear increase in the incorporation of these fatty acids into membrane lipids in the developing infant (Carlson et al. 1986 and 1987) with a concomitant decrease in 20:4(n-6) levels.

Docosahexaenoic acid (DHA)⁴ is necessary for normal neural and visual development (Neuringer et al. 1988). When preterm infants were fed formula fortified with marine oil, their 22:6(n-3) status improved, and the rate of development of early visual acuity was accelerated (Carlson et al. 1989). However, 20:4(n-6) levels in phospholipids declined and the infants supplemented with only marine oil grew less well than those fed standard preterm formula (Carlson et al. 1992a, 1992b, 1992c, 1993 and 1996). When the formula contained both 20:4(n-6) and 22:6(n-3), no apparent differences in growth were observed (Clandinin et al. 1992 and 1997b, Koletzko et al. 1995). However, it should be noted that an insufficient number of infants were studied to test for small effects on growth.

Feeding lactating rats diets with or without preformed 20:4(n-6) and 22:6(n-3) markedly alters the phospholipid fatty acid composition in the developing brain of the rat pup (Jump-

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⁴ Abbreviations used: AA, arachidonic acid; ACTH, adrenal corticotrophic hormone; CG, chorionic gonadotropin; DHA, docosahexaenoic acid; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; PL, placental lactogen; PRL, prolactin; TSH, thyroid-stimulating hormone.

TABLE 1

Composition of the experimental diets¹

Ingredient	
	<i>g/kg of diet</i>
High protein casein	270
Cornstarch	200
Glucose	207.65
Fat	200
Nonnutritive cellulose	50
Vitamin mix ²	10
Mineral mix ³	50.85
Choline	2.75
Inositol	6.25
L-Methionine	2.50

¹ The complete nutrient composition of this basal diet has been reported earlier (Clandinin and Yamashiro 1982) and meets AIN 1993 recommendations when essential nutrient content is calculated for a high fat diet.

² AOAC vitamin mix (Teklad Test Diets, Madison, WI).

³ Bernhart-Tomarelli mineral mix (General Biochemicals, Chagrin Falls, OH) was modified to provide 77.5 mg of Mn²⁺ and 0.06 mg Se²⁺ per kilogram of complete diet.

sen et al. 1995, 1996 and 1997). These changes in membrane composition vary between brain regions and the timing in development at which they occur, and are different when neuronal and glial cell types are compared (Jumpsen and Clandinin 1995).

The anterior hypophysis releases a variety of hormones, among them thyroid-stimulating hormone (TSH), adrenal corticotrophic hormone (ACTH), growth hormone (GH) and prolactin (PRL), that have a pervasive effect on the growth, differentiation and function of target organs throughout the body. Indeed, GH is synergistic with ACTH in increasing adrenal size, affecting reproductive organ development with androgens and increasing body protein content. Neuroregulation of the anterior pituitary arises from the portal hypophyseal

TABLE 2

Fatty acid composition of diets fed¹

Fatty blend	Control	AA ²	DHA	AA and DHA
g/100 g fatty acids				
12:0	9.6	7.3	10.2	7.9
14:0	5.8	4.8	6.2	5.7
14:1	0.3	0.1	0.1	0.2
16:0	12.7	15.7	14.7	15.9
16:1	1.3	1.2	1.2	1.4
18:0	7.7	9.3	7.3	8.9
18:1(n-9)	40.7	38.9	38.3	38.8
18:2(n-6)	15.8	16.2	16.0	16.8
18:3(n-3)	2.2	2.3	2.3	2.3
20:0	0.3	0.3	—	0.3
20:1(n-9)	0.2	0.5	0.4	0.5
20:4(n-6)	—	1.2	—	1.2
20:5(n-3)	—	—	—	—
22:6(n-3)	—	0.1	0.8	0.7

¹ Four fat blends were formulated: control, control + AA, control + DHA and control + AA + DHA. These blends were added at 200 g/kg of basal diet indicated in Table 1. The control blend was designed to reflect the fatty acid composition of current infant formula fat blend and an (n-6)/(n-3) fatty acid ratio of 7.3 to 1.

² AA, arachidonic acid; DHA, docosahexaenoic acid.

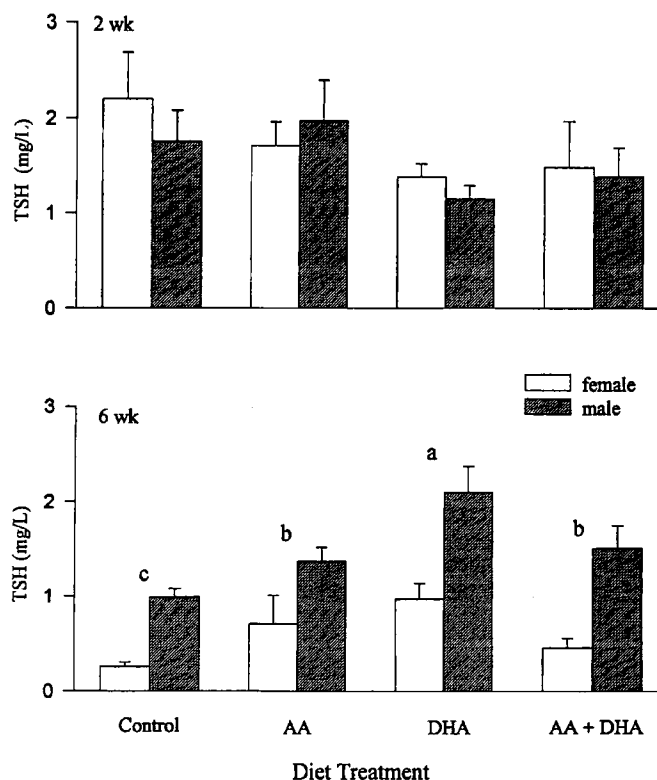


FIGURE 1 Plasma thyroid-stimulating hormone (TSH) concentrations in rats fed diets with or without arachidonic acid (AA), docosahexaenoic acid (DHA) or AA + DHA. At 6 wk of age, significant effects of diet treatment are indicated for male rats by different superscripts (a, b, c) ($P < 0.05$). Male vs. female, $P < 0.01$. Blood was drawn from rat pups at the time periods indicated. Values are means $\pm$ SD, $n = 10$.

vessels. In our previous studies of suckling rat pups (Jumpsen et al. 1996 and 1997), it was not feasible to dissect the pituitary and measure membrane changes that may have occurred in various cell types in response to feeding 20:4(n-6) or 22:6(n-3). However, it is logical to hypothesize that feeding these fatty acids may also affect the composition of neuronal membranes in the anterior and posterior regions of the pituitary. These types of diet-induced changes in membrane composition occurred in other brain regions. If analogous change in membrane composition occurs in the pituitary, it is reasonable to hypothesize that such changes in membrane structure will affect the function of this endocrine organ and perhaps the levels of hormones secreted from the pituitary that affect growth at different stages of neural development.

This study was designed to assess the circulating levels of hormones released from the anterior pituitary (TSH, GH, ACTH and PRL) in suckling rats and young rats in the post-weaning period after they were fed diet fats that reflect the fat composition of a current infant formula with or without the addition of 20:4(n-6) and 22:6(n-3).

MATERIALS AND METHODS

Animal breeding. This research protocol was reviewed by the University Animal Care Committee and complies with the NIH guidelines (NRC 1985). Sprague-Dawley rats were bred in the laboratory for the study. All rats were housed in plastic cages fitted with stainless steel mesh covers in a temperature- and humidity-controlled room with a 12-h light:dark cycle. As soon as the pregnant dams delivered, they were given free access to one of the test diets. Litter size was equalized at 12 pups and assignment of dams and litters to

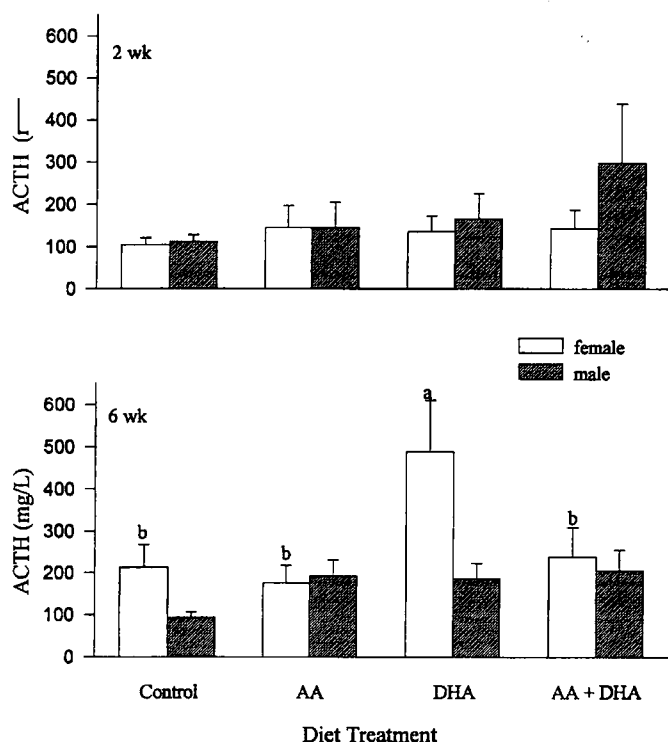


FIGURE 2 Plasma adrenal corticotrophic hormone (ACTH) levels in rats fed diets with or without arachidonic acid (AA), docosahexaenoic acid (DHA) or AA + DHA. There were no significant effects of treatment at 2 wk of age. At 6 wk of age, significant effects of diet treatment are indicated for females by different superscripts (a, b). Blood was drawn from rat pups at the time periods indicated. Values are means $\pm$ SD, $n = 10$.

treatment was random. A minimum of five litters were assigned to each diet treatment for each value illustrated at each age. Litters were used at 2 wk of age for blood collection and subsequent hormone analyses. Other litters were suckled until 3 wk of age, at which time the pups were weaned and given free access to the same diet as their mother. These litters were used at 6 wk of age for blood collection and hormone analyses.

Diets. The semipurified diets fed contained 20 g/100 g total fat (Table 1) (Clandinin and Yamashiro 1982). The diet fat mixture was formulated to reflect the fatty acid composition of a current infant formula fat blend, the control blend (SMA, Wyeth-Ayerst Laboratories, Radnor, PA) or modified to provide levels of 20:4(n-6) or 22:6(n-3) that could be incorporated into a potentially new infant formula fat blend [arachidonic acid (AA), DHA or AA + DHA; Table 2], reflecting the content of these fatty acids in human milk. Both 20:4(n-6) and 22:6(n-3) were provided in the fat blend as a triglyceride.

Blood collection. All blood samples were rapidly collected (i.e., within 30 s) by cardiac puncture in tubes after brief exposure to halothane at the same time of the morning. Blood was then centrifuged at 4°C (1855 $\times$ g for 10 min). The serum-plasma was removed, transferred to a microcentrifuge tube, immediately placed on dry ice and stored at -70°C under N₂ until analyzed. Samples at 2 and 6 wk of age were analyzed in a batch a few days after sample collection.

Hormone analysis. TSH, GH, ACTH and PRL were analyzed by RIA procedures (TSH and PRL, Ciba Corning Canada, Markham, Canada; GH, Diagnostics Products, Los Angeles, CA; ACTH, Nichols Institute Diagnostics, San Juan Capistrano, CA). All hormones were analyzed for each rat. These assays were tested with rodent hormone as described in the following.

TSH was measured by using a Ciba-Corning TSH diagnostic kit (Ciba Corning Canada). The Ciba Corning TSH assay is a two-site chemiluminometric immunoassay, which uses constant amounts of two antibodies. The first antibody or Lite Reagent is a monoclonal

mouse anti-TSH antibody labeled with an acridinium ester. The second antibody or Solid Phase is a polyclonal sheep anti-TSH antibody covalently coupled to a paramagnetic particle. A direct relationship exists between the TSH in a sample and the relative light units detected by the ACO:180 analyzer. The TSH assay measures TSH concentrations up to 150 mIU/L with a minimum detectable concentration of 0.03 mIU/L. The cross-reactivity of the TSH assay with luteinizing hormone (LH), follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) has been determined by adding these hormones to serum controls containing TSH. The level of TSH of the serum was not affected.

Rat serum PRL level was assayed using a Ciba Corning PRL kit (Ciba Corning Canada). PRL assay is a two-site chemiluminometric (sandwich) immunoassay, similar to the TSH assay. A direct relationship exists between the PRL in the serum sample and relative light units (RLUs) detected by the ACS:180 system. The prolactin assay measures prolactin concentrations up to 200 μ g/L with a minimum detectable concentration of 0.3 μ g/L. The cross-reactivity of the PRL assay with TSH, LH, hCG, FSH, human growth hormone (hGH) and human placental lactogen (hPL) was determined by adding these hormones to serum samples containing PRL. The presence of TSH, LG, hCG and FSH slightly increased the PRL level in the serum samples but hGH and hPL had no effect.

The GH level was measured by a double antibody hGH diagnostic kit, manufactured by Diagnostic Products. In the double antibody hGH procedure, ¹²⁵I-labeled GH competes with GH in serum or plasma for sites on the GH specific antibody. After incubation for a fixed time, separation of bound from free is achieved by the PEG-accelerated double-antibody method. The tube is then counted in a gamma counter; the counts are inversely related to the amount of GH present in the sample. By the more conservative definition, the GH assay has a detection limit of $\sim$ 0.9 μ g/L using the standard 2-h room-temperature procedure. The antiserum is highly specific for GH with the exception of PRL, which has a molecular structure similar

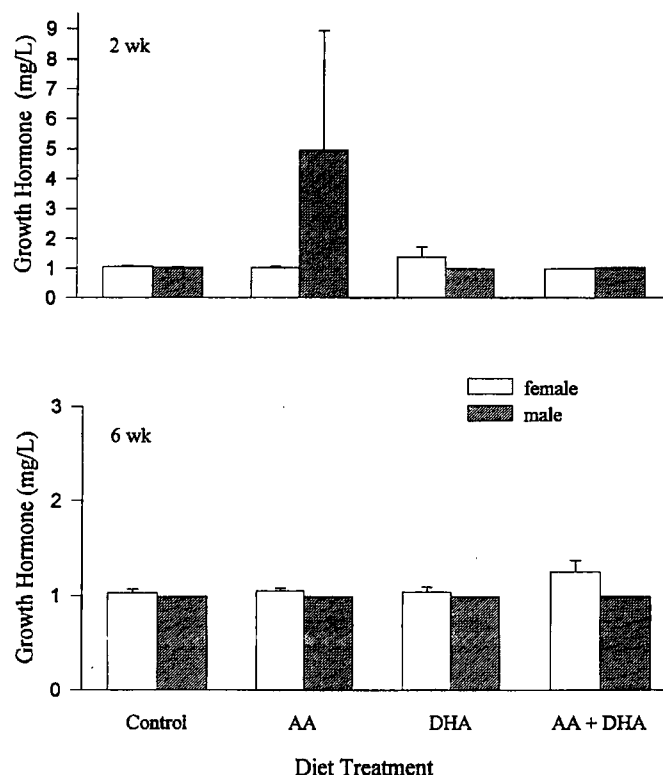


FIGURE 3 Plasma growth hormone concentrations in rats fed diets with or without arachidonic acid (AA), docosahexaenoic acid (DHA) or AA + DHA. These values are just above detection limits. There were no significant effects of treatment. Blood was drawn from rat pups at the time periods indicated. Values are means $\pm$ SD, $n = 10$.

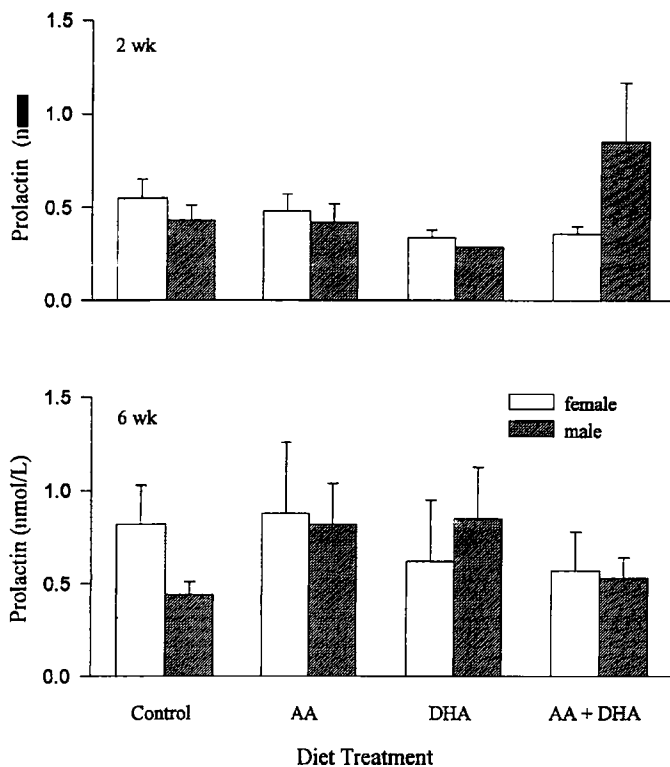


FIGURE 4 Plasma prolactin concentrations in rats fed diets with or without arachidonic acid (AA), docosahexaenoic acid (DHA) or AA + DHA. There were no significant effects of treatment. Blood was drawn from rat pups at the time periods indicated. Values are means $\pm$ SD, $n = 10$.

to that of GH. The antiserum exhibits low cross-reactivity to other hormones.

For the quantitative determination of ACTH in plasma, an ACTH 65T kit (Nichols Institute Diagnostics) was used. The ACTH immunoassay incorporates a monoclonal antibody and a polyclonal antibody, both with high affinity and specificity for defined amino acid regions of the ACTH molecule. The monoclonal antibody is radiolabeled for detection, whereas the polyclonal antibody is coupled to biotin. The addition to the reaction mixture of an avidin-coated plastic bead allows for a specific and efficient means of binding the sandwich complex via the high affinity interaction between biotin and avidin. In the assay system, standards, controls and samples are incubated with a solution containing both the radiolabeled antibody and biotin-coupled antibody, and an avidin-coated plastic bead. At the end of the assay incubation, the bead is washed to remove unbound components, and the radioactivity bound to the solid phase is measured in the gamma counter. A dose-response curve of radioactivity vs. concentration is generated by using results obtained from standards that are assayed concurrently with the unknowns. Concentrations of intact ACTH in the controls and samples are determined directly from the curve. The highest concentration of ACTH measurable without dilution is the value of the highest standard (20,000 ng/L) and the lowest measurable concentration is 1.0 ng/L.

Statistical analysis. Each value illustrated is based on at least 10 replicates from a minimum of five different litters. One rat was considered a replicate. The effect of treatment was determined by three-way ANOVA procedures followed by a Duncan's multiple range test (Steel and Torrie 1980) when a significant effect of diet was observed. The sum of squares used was the error mean square.

RESULTS AND DISCUSSION

Analyses of the stomach contents of the suckling rat pups at 2 wk of age indicated that a substantial amount of the

20:4(n-6) and 22:6(n-3) added to the dam's diet was transferred into the milk (data not shown). Addition of 1.2 g/100 g 20:4(n-6) or 0.7 g/100 g 22:6(n-3) increased the level of these fatty acids in the milk approximately two- to threefold and three- to sixfold, respectively (Jumpsen and Clandinin 1996). All groups exhibited similar weight gains (data not illustrated).

At 2 wk of age, no effect of diet on circulating levels of TSH (Fig. 1), ACTH (Fig. 2), GH (Fig. 3) or PRL (Fig. 4) was apparent. By 3 wk postweaning, 6-wk-old male pups consuming the diet containing 22:6(n-3) had significantly elevated levels of TSH and female pups had greater levels of ACTH. No effect of diet was observed on circulating GH or PRL levels. Male pups had higher levels of TSH than females (< 0.0001 ; Fig. 1), whereas female pups from the 22:6(n-3) diet treatment had significantly higher concentrations of ACTH than all male pups.

Growth is a function of many factors that can be limited by restricting the availability of essential nutrients and by metabolic interactions that may also serve to limit the availability of an essential precursor. Both 20:4(n-6) and 22:6(n-3) are essential components of the membrane structural lipids (Clandinin et al. 1991 and 1994). It is therefore logical that a decrease in the status of one of these fatty acids, as measured by a variety of potential dynamic metabolic markers [for example, levels in plasma phospholipid pools, erythrocyte phosphatidylcholine, plasma triglycerides (Carlson et al. 1992c and 1993, Koletzko and Braun 1991)], may be reflected in a decrease in cell growth in some tissues. At present, however, it is hard to envisage how a change in these metabolic markers will be reflected in changes in gross body weight or the composition of growth. Moreover, it is not known which tissues or cell types are the first to be affected when essential fatty acid status declines to a critical or limiting level.

If similar changes occur in pituitary function in the human infant, the results of this study are suggestive of an entirely different basis for the differences in growth observed after varying intakes of (n-6) and (n-3) fatty acids in infant feedings. Is it reasonable to speculate that infants supplemented with fish oil containing 20:5(n-3) and 22:6(n-3) may have elevated TSH levels? It is also noteworthy that small preterm infants have a fragile capability to produce corticosteroids and are often given corticosteroids to stimulate phosphatidylcholine production for surfactant. Perhaps this need for corticosteroid is also a function of the infant's essential fatty acid status affecting ACTH release. The observations of this study, although not conclusive in terms of demonstrating a clear effect of diet on preweaning to postweaning growth, are suggestive of an effect of early diet on developing neuroendocrine regulatory mechanisms.

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Dietary Fish Oil and Cytochrome P-450 Monooxygenase Activity in Rat Liver and Kidney

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ABSTRACT: Lauric acid hydroxylation and aminopyrine *N*-demethylation were studied in kidney and liver microsomes from rats treated with fish oil. Different doses of fish oil containing 20% eicosapentaenoic acid and 10% docosahexaenoic acid were provided daily to the rats for seven days. In all the groups studied, the lauric acid metabolism was higher in kidney microsomes and the aminopyrine metabolism in the liver microsomes. Although no effect on the renal cytochrome P-450 concentration was detectable, all four fish oil doses increased the hepatic concentration of cytochrome P-450 by a mean 27%. The higher fish oil doses used increased the renal and hepatic microsomal metabolism of aminopyrine. The lauric acid metabolism was increased by fish oil only in the liver. Fish oil, a known inducer of fatty acid peroxisomal β -oxidation, also induced microsomal activity. These results show that liver and kidney respond in different ways to dietary factors such as fish oil. In addition, our study would suggest that fish oil increased the activity of two different families of liver cytochrome P-450. The activity of kidney lauric acid 11- and 12-hydroxylation, however, was not modulated by fish oil. *Lipids* 30, 955–958 (1995).

Microsomal cytochrome P-450 catalyzes the oxidative metabolism of a wide variety of lipophilic compounds, such as drugs, carcinogens, endogenous steroids (1), and fatty acids (2). Cytochrome P-450s are found predominantly in the liver, but are also present in many other tissues, such as the kidney.

Marine oils are rich in polyunsaturated fatty acids of *n*-3 family, especially eicosapentaenoic acid (20:5) (EPA) and docosahexaenoic acid (22:6) (DHA). There are several cytochrome P-450 isoenzymes from the family 4A that oxidize fatty acids. The CYP4A1 purified from liver of clofibrate-treated rats catalyzes the ω -hydroxylation of arachidonic and lauric acids (3). The physiological role of these isoenzymes P-450 has not been identified.

It has been reported that clofibrate and other peroxisomal proliferators simultaneously enhance the cellular content and activity of liver cytochrome P-450 (3). In previous studies with starved rats, we showed an increase in the microsomal

lauric acid ω -hydroxylation, which was correlated with an increase in peroxisomal fatty acid β -oxidation in liver and in kidney (4,5).

The liver microsomal activity has been studied extensively. Meanwhile, our knowledge of both the constitutive and inducible content and activity of kidney cytochrome P-450 remains very limited. The levels of cytochrome P-450 in kidney previously have been shown to be much lower than those seen in liver, and the renal cortex presents the highest levels of cytochrome P-450 (6,7).

It has been reported that fish oil or diets enriched in EPA and DHA induce liver fatty acid microsomal and peroxisomal oxidation (8–11). The extent of the increase of microsomal activity or peroxisomal β -oxidation depends on the type of dietary fish oil (10). However, the effect of fish oil on kidney microsomal activity has not been reported.

In this work, we study the effect of fish oil consumption on microsomal lauric acid hydroxylation and aminopyrine *N*-demethylation to investigate the possible induction of the microsomal activity by this factor. We carried out a comparative study using liver and kidney microsomes in order to determine how these tissues respond to a diet enriched with polyunsaturated fatty acids EPA and DHA.

MATERIALS AND METHODS

Chemicals. Fish oil was provided by Maver Laboratory (Santiago, Chile). According to the manufacturers, the fish oil was encapsulated under vacuum using anerobic conditions. The capsules were stored at room temperature upon arrival and opened just prior to the rat gavages. The fish oil was a sardine oil concentrate containing 36.5% polyunsaturated fatty acids [20.4% EPA, 10.3% DHA, 1.4% linoleic, 0.8% linolenic, 1.6% octadecatetraenoic (18:4n-3), 0.4% eicosadienoic (20:2n-9), 1.1% arachidonic, and 0.5% docosatetraenoic acids (22:4n-6)]; 32.8% monounsaturated fatty acids [12.4% palmitoleic, 16% oleic, 2.5% eicosanoic (20:1n-9), and 1.9% cetoleic acids], and 30.7% saturated fatty acids (0.7% pentadecanoic, 20.1% palmitic, 1.0% heptadecanoic, 4.1% tetra-cosanoic, and 4.8% stearic acids). The corn oil used in our control rats was provided by Mazola (Inducoen, Santiago, Chile), and contained 60% total polyunsaturated fatty acids (57.7% linoleic and 2.3% linolenic acids); 26.1% of the mo-

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Abbreviations: ANOVA, analysis of variance; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

nounsaturated oleic acid, and 13.6% saturated fatty acids (10.7% palmitic, 2.8% stearic, and 0.1% docosanoic acids). Lauric acid, aminopyrine, NADPH, isocitrate dehydrogenase, and sodium isocitrate were purchased from Sigma Chemical Co. (St. Louis, MO). (1-¹⁴C) Lauric acid (56 mCi/mmol) was from Amersham (Arlington Heights, IL). All the other chemicals were obtained from commercial sources and were of the highest purity available.

Animals and treatments. Mature male Wistar rats (Charles River Breeding Laboratories, North Wilmington, MA) (weighing 200–250 g) were used throughout. Rats were fed for seven days with either pure corn oil or the fish oil described previously (0.5–7.5 g/kg body weight). The lowest fish oil dose used was comparable to that used clinically in Chile. The daily oil supplementation was given by gastric tube feeding, and an equivalent volume was applied to control and to treated rats (0.1–1.5 mL). All the animals had free access to standard pellets and water.

Microsomal activities. Microsomes from whole kidney or liver were prepared as described elsewhere (12). Decapsulated kidneys or livers of 3–5 rats were used for each microsomal sample. The total cytochrome P-450 content was measured as described by Omura and Sato (13). Sodium succinate was added to kidney microsome preparations to minimize hemoprotein interferences in light transmission, which become significant in tissues with low cytochrome P-450 content (14).

Lauric acid hydroxylation. This activity measurement was performed according to the method described previously (4). Lauric acid and its 11-hydroxy and 12-hydroxy derivatives were resolved by a reverse-phase high-performance liquid chromatographic technique as previously described (5).

Aminopyrine N-demethylation. The measurement of oxidative demethylation was performed according to the method described by Nash (15). The reaction mixture contained 10 mM aminopyrine, 50 mM buffer Tris-Cl, pH 7.5, 150 mM KCl, 10 mM MgCl₂, 8 mM sodium isocitrate, 0.25 IU/mL isocitrate dehydrogenase, 1 mM NADP, and 3.0 mg/mL of kidney microsomal protein. Incubations were performed at 37°C for a 20-min period (a range where the reaction was linear with time). Results are expressed as means ± SD, and the statistical significance of differences between mean values was assessed by analysis of variance (ANOVA) coupled with Student-Newman-Keuls test at the 5% level of significance.

RESULTS

Table 1 shows the different doses of fish oil provided to the rats daily for seven days, using 0.5 g/kg body weight initially and then increasing to 7.5 g/kg body weight. The body and kidney weight were not significantly altered by fish oil (220 ± 10 and 1.09 ± 0.10 g, respectively). The liver weight, however, increased about 20% in all the groups treated. The kidney microsomal protein was not significantly altered by fish oil (7.5 ± 0.81 mg/g kidney); in contrast, an increase of about 27% in liver microsomal protein was observed with all the

TABLE 1
Effect of Different Doses of Fish Oil on the Liver Weight, Microsomal Protein, and Cytochrome P-450 Content

Group	Fish oil (g/kg) ^a	Liver weight (g) ^b	Microsomal protein (mg/g liver) ^c	Cytochrome P-450 (nmol/g liver) ^c
Control	0.00	11.8 ± 1.10	9.1 ± 1.02	5.28 ± 0.6
Dose I	0.50	14.4 ± 1.50 ^d	11.8 ± 1.10 ^d	7.08 ± 0.8 ^d
Dose II	1.00	14.0 ± 1.60 ^d	11.6 ± 0.90 ^d	6.38 ± 0.6
Dose III	5.00	14.2 ± 0.50 ^d	11.9 ± 1.30 ^d	7.14 ± 1.1 ^d
Dose IV	7.50	14.8 ± 1.50 ^d	11.0 ± 1.40 ^d	6.27 ± 0.6

^aFish oil containing 20% eicosapentaenoic acid and 10% docosahexaenoic acid was provided daily in the doses indicated during seven days, and are expressed as g oil/kg body weight.

^bValues are the means ± SD of at least 20 rats.

^cValues are the means ± SD of at least five different experiments.

^dSignificantly different from control ($P < 0.05$) as assessed by analysis of variance coupled with Student-Newman-Keuls test.

doses used (Table 1). It is known that the kidney content of cytochrome P-450 is lower than that of the liver (6,7). In the control rats, the cytochrome P-450 concentration in kidney (0.98 ± 0.24 nmol/g wet weight) was one-fifth that found in liver (5.28 ± 0.60). Although no effect on the renal cytochrome P-450 concentration was detectable, all four fish oil doses increased the hepatic concentration of cytochrome P-450 by 27% (Table 1). Because the dietary fish oil also increased the liver size by 22%, all four doses of fish oil increased the total liver microsome P-450 content by about 55% (i.e., from 62.3 ± 18.1 nmol cytochrome P-450/liver to 101.95 ± 20.4 control and dose I values, respectively). Surprisingly, the effects on liver weight, the cytochrome P-450 concentration, and content did not increase with the fish oil dosage. There were no significant differences in the liver weight, microsomal protein, and cytochrome P-450 content between the fish oil doses I–IV, as assessed by ANOVA coupled with the Student-Newman-Keuls test.

The effect of fish oil on liver microsomal metabolism of lauric acid is shown in Table 2. Fish oil had no effects on renal microsome capacity to ω- and ω1-hydroxylate lauric acid, which remained at 0.38 ± 0.08 and 0.72 ± 0.10 nmol product/min/mg protein, respectively. In liver, however, a significant increase of about 43% was seen in the overall rate of

TABLE 2
Effect of Fish Oil on the Lauric Acid Hydroxylation Catalyzed by Rat Liver Microsomes^a

	Metabolites (nmol product/min/mg protein)		
	11-Hydroxy lauric acid	12-Hydroxy lauric acid	Total
Control	0.50 ± 0.13	0.95 ± 0.16	1.44 ± 0.25
Dose I	0.52 ± 0.12	1.11 ± 0.25	1.63 ± 0.40
Dose II	0.44 ± 0.10	0.87 ± 0.12	1.31 ± 0.18
Dose III	0.82 ± 0.05	1.25 ± 0.10	2.07 ± 0.28 ^b
Dose IV	0.67 ± 0.12	1.38 ± 0.15	2.05 ± 0.29 ^b

^aValues are the means ± SD of at least five different experiments.

^bSignificantly different from control ($P < 0.05$) as assessed by analysis of variance coupled with Student-Newman-Keuls test.

TABLE 3
Effect of Fish Oil on the Aminopyrine N-Demethylation
Catalyzed by Rat Kidney and Liver Microsomes^a

Group	(nmol product/min/mg protein)	
	Kidney	Liver
Control	0.06 ± 0.01	1.53 ± 0.21
Dose III	0.17 ± 0.02 ^b	2.96 ± 0.13 ^b
Dose IV	0.23 ± 0.05 ^b	3.34 ± 0.36 ^b

^aValues are the means ± SD of at least five different experiments.

^bSignificantly different from control ($P < 0.05$) as assessed by analysis of variance coupled with Student-Newman-Keuls test.

lauric acid hydroxylation with the highest fish oil doses used (5 and 7.5 g/kg body weight). Only the effect of fish oil dose II was significantly different from doses III and IV. Because the fish oil also increased the amount of microsomal protein by an average of 27% (Table 1), the total hydroxylase activity in liver increased from a control value of 13.10 ± 1.44 nmol product/min/g liver to 19.23 ± 2.12 , 15.20 ± 1.50 , 24.63 ± 5.21 , and 22.55 ± 5.24 , with doses I, II, III, and IV, respectively. Thus, fish oils increased both the total cytochrome content and the total lauric acid hydroxylase activity in liver. In all the groups studied, the metabolite produced in a major proportion was the 12-hydroxy lauric acid (ω -derivative).

Aminopyrine N-demethylation was assayed in order to study the effect of fish oil on the activity of a different cytochrome P-450 family than CYP4A. Only fish oil doses III and IV were used in these studies. The renal metabolism of aminopyrine was lower than that of the hepatic tissue, the control group being about 4% of the liver values (Table 3). The metabolism of this exogenous substrate was significantly increased in both organs in rats treated with the higher doses of fish oil. The aminopyrine metabolism increased by about 3–4-fold in kidney and about 2-fold in liver in respect to the controls. There were significant differences between the effect of fish oil doses III and IV in both organs.

The liver and kidney microsomal turnover rates for aminopyrine and lauric acid was compared. In all the groups studied, the renal turnover rate of aminopyrine was lower than the hepatic one, being about 17% of the liver values in the control group (0.46 ± 0.07 and 2.65 ± 0.08 nmol product/min/nmol cytochrome P-450 for the kidney and liver values, respectively). In contrast, the kidney exhibited a higher metabolism of lauric acid. The turnover rate (nmol lauric acid product/min/nmol cytochrome P-450) in kidney (8.46 ± 2.01) was about threefold that in liver (2.48 ± 0.40).

DISCUSSION

In our studies, the liver and kidney respond in a different way to fish oil ingestion. Whereas the aminopyrine metabolism in both organs was increased by fish oil, the lauric acid metabolism increased only in the liver microsomes. Little is known about the fish oil effect on the kidney microsomal activity. In contrast to liver, kidney cytochrome P-450 is poorly induced by phenobarbital and clofibrate (16,17). Also, the response to

peroxisome proliferators, such as di(ethylhexyl)phthalate, is different in liver and in kidney (18).

In relation to the effect of fish oil on the liver microsomal lauric acid metabolism, our results are similar to those obtained by Nilsson *et al.* (19). By feeding rats a diet with 20% fish oil (w/w) for one week, they obtained a more acute effect in increasing the liver lauric acid metabolism (about 2.5-fold compared to controls). The difference between our results and those reported by Nilsson *et al.* (19) might be attributed to the different fish oil origin or the doses ingested. Mounie *et al.* (20), by feeding rats a diet with a 6% fish oil content, reported an increased cytochrome P-450 content and bilirubin glucuronosyl transferase activity in liver.

Part of the clinical properties attributed to EPA (20:5n-3) are due to its structural similarity to arachidonic acid (20:4n-6) (21,22). Cytochrome P-450 participates in the metabolism of arachidonic acid, generating ω -derivates, epoxides, and others metabolites with biological activity (2,23). Several cytochrome P-450 isoenzymes involved in the metabolism of arachidonic acid have been purified in the rat liver and kidney (24). With the increasing interest in the health benefits of marine oils containing n-3 polyunsaturated fatty acids, it has been shown that EPA and DHA also can be metabolized by cytochrome P-450 *in vitro* (25). Urinary excretion of epoxides and diols derived from EPA during the fish oil ingestion by humans has been reported (26). In the microsomal metabolism of EPA, the participation of CYP4A family, specifically to metabolize fatty acids in the ω -position, is probable.

It is known that the levels and activities of cytochrome P-450 enzymes are affected by the diet (1,2). Our studies show that the liver and kidney respond in a different way to a dietary factor such as fish oil. The fish oil increased the lauric acid metabolism only in the liver. The aminopyrine metabolism, however, was induced in both organs. These results suggest that fish oil increases the activity of two different liver cytochrome P-450 families. The activity of kidney lauric acid 11- and 12-hydroxylase, however, was not modulated by this factor. More experimental work is needed to explore the biological significance of these selective changes.

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[Received December 29, 1994; Revision accepted August 31, 1995]



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740

Dr. Matthias Liebergesell
DuPont Applied BioSciences
Route 141 & Powder Mill Road,
Wilmington, Delaware 19803-0356

JUN 16 2009

Dear Dr. Matthias Liebergesell :

This is to inform you that the notification, dated March 31, 2009 which you submitted pursuant to 21 U.S.C. 350b(a)(2)(section 413(a)(2) of the Federal Food, Drug, and Cosmetic Act (the Act)) was received and filed by the Food and Drug Administration (FDA) on April 3, 2009. Your notification concerned "EPA-rich triglyceride oil [and] EPA refers to Eicosapentanoic acid" that you identify as a new dietary ingredient which is obtained through a fermentation process using *Yarrowia lipolytica*.

According to your notification, the conditions of use for your new dietary ingredient was stated to be "consumption of up to 2 grams (g) of EPA per day would be recommended or suggested in the labeling of the dietary supplement. Thus, use of DuPont's EPA-rich triglyceride oil in accordance with label recommendations would result in a maximum EPA intake of 2 g/day."

Under 21 U.S.C. 350b(a), the manufacturer or distributor of a dietary supplement containing a new dietary ingredient that has not been present in the food supply as an article used for food in a form in which the food has not been chemically altered must submit to FDA, at least 75 days before the dietary ingredient is introduced or delivered for introduction into interstate commerce, information that is the basis on which the manufacturer or distributor has concluded that a dietary supplement containing such new dietary ingredient will reasonably be expected to be safe. FDA reviews this information to determine whether it provides an adequate basis for such a conclusion. Under 21 U.S.C. 350b(a)(2), there must be a history of use or other evidence of safety establishing that the new dietary ingredient, when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe. If this requirement is not met, the dietary supplement is considered to be adulterated under 21 U.S.C. 342(f)(1)(B) because there is inadequate information to provide reasonable assurance that the new dietary ingredient does not present a significant or unreasonable risk of illness or injury.

FDA-1995-S-0039-0260

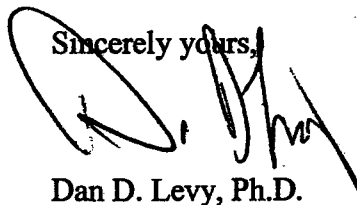
RPT 582

This letter is to alert you that FDA intends to complete its evaluation within a few weeks and send you a response to your notification after we receive the additional information related to the safety studies you conducted as well as more information on serving levels and conditions of use for your ingredient (as discussed in a telephone conference of June 8, 2009). Please note that a lack of a response to a notification within the 75-day timeframe does not constitute a finding by the agency that the ingredient or a product that contains the ingredient is safe or is not adulterated under 21 U.S.C. 341. See 21 C.F.R. 190.6 (f).

Your notification will be kept confidential for 90 days after the filing date of April 3, 2009. After the 90-day date, the notification will be placed on public display at FDA's Docket Management Branch in docket number FDA-1995-S-0039 (formerly docket number 95S-036). The FDA received on May 28, 2009, the documentation in which you identified in writing the specific information you believe is proprietary, trade secret or otherwise confidential for FDA's consideration for your original notification. You may wish to identify in writing that information you consider proprietary, trade secret or otherwise confidential for the material you are currently providing to the FDA for us to complete our review.

If you have any questions concerning this matter please contact Dr. Fred Hines, Consumer Safety Officer for the New Dietary Ingredient Review Team, at (301) 436-1756.

Sincerely yours,

A handwritten signature in black ink, appearing to read 'Dan D. Levy', is written over the typed name.

Dan D. Levy, Ph.D.
Senior Microbiologist, Acting Supervisor
New Dietary Ingredient Review Team
Division of Dietary Supplement Programs
Office of Nutrition, Labeling and Dietary Supplements
Center for Food Safety and Applied Nutrition



Experimental Station
Rt. 141 & Powder Mill Road
Wilmington, DE 19880-0356

May 26, 2009

Fred A. Hines DVM
Consumer Safety Officer (HFS-810)
Division of Dietary Supplement Programs
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Harvey W. Wiley Building
5100 Paint Branch Parkway
College Park, MD 20740-3835

FAH/FDA
MAY 28 2009
NDI 582

RE: New Dietary Ingredient Notification for DuPont's EPA-rich triglyceride oil;
Proprietary, trade secret or otherwise confidential information

Dear Dr. Hines:

We respectfully submit a letter identifying confidential and propriety information and their location(s) within the document, pursuant to Section 413(a) of the Federal Food, Drug, and Cosmetic Act.

The confidential and proprietary information is related to the EPA-rich triglyceride oil chemical composition, manufacturing, and specifications, as well as discussion and results of unpublished data.

The identified trade secrets and otherwise confidential commercial information should not be disclosed to the public pursuant to 21 C.F.R. § §20.61 and 190.6(e).

Respectfully,

A handwritten signature in black ink, appearing to read "M. Liebergesell", written in a cursive style.

Dr. Matthias Liebergesell
Global Regulatory Affairs Manager
DuPont Applied BioSciences

SECTION 1

The name and complete address of the manufacturer or distributor of the dietary supplement that contains a new dietary ingredient, or of the new dietary ingredient.

The manufacturer of the new dietary ingredient is:

E.I. du Pont de Nemours and Company

Direct correspondence to:

Dr. Matthias Liebergesell
Regulatory Affairs Manager
DuPont Applied BioSciences
Experimental Station 356/244B
Rt. 141 & Powder Mill Road
Wilmington, DE 19803-0356
Tel.: 302-695-7103
Fax: 302-695-7056
Matthias.Liebergesell@usa.dupont.com

SECTION 2

The name of the new dietary ingredient.

EPA-rich triglyceride oil. EPA refers to Eicosapentaenoic acid.

SECTION 3

Description of the dietary supplement or dietary supplements that contain the dietary ingredient including (i) the level of the dietary ingredient in the dietary supplement, and (ii) the conditions of use recommended or suggested in the labeling of the dietary supplement, or if no conditions of use are recommended or suggested in the labeling of the dietary supplement, the ordinary conditions of use of the supplement.

DuPont's EPA-rich triglyceride oil is intended for use in dietary supplements to provide omega-3 polyunsaturated fatty acids, and specifically, eicosapentaenoic acid (EPA). It contains natural tocopherols and other safe and suitable components for stabilization and will be sold in forms suitable for dietary supplements. Consumption of up to 2 grams (g) of EPA per day would be recommended or suggested in the labeling of the dietary supplement. Thus, use of DuPont's EPA-rich triglyceride oil in accordance with label recommendations would result in a maximum EPA intake of 2 g/day.

SECTION 4

The history of use or other evidence of safety establishing that the dietary ingredient, when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe, including any citation to published articles or other evidence that is the basis on which the distributor or manufacturer has concluded that the dietary supplement will reasonably be expected to be safe.

DuPont intends to produce a triglyceride oil that has a high content of omega-3 fatty acids, specifically, more than 35% of the triglyceride oil is EPA. The oil is obtained through a fermentation process using *Yarrowia lipolytica*, a yeast with a history of safe use as a food production microorganism. Information related to the production of the oil is provided in Sections 4.1 through 4.5.

4.1 *Yarrowia lipolytica* Characterization

4.1.1 Identification/taxonomic classification

- *Yarrowia lipolytica* ATCC #20362 (Wickerham *et al.*) van der Walt et von Arx, teleomorph; deposited by Biotechnika Int'l. as *Candida lipolytica* (Harrison) Diddens et Lodder, anamorph
- Biosafety Level: 1

4.1.2 Occurrence in Nature and History of Commercial Use

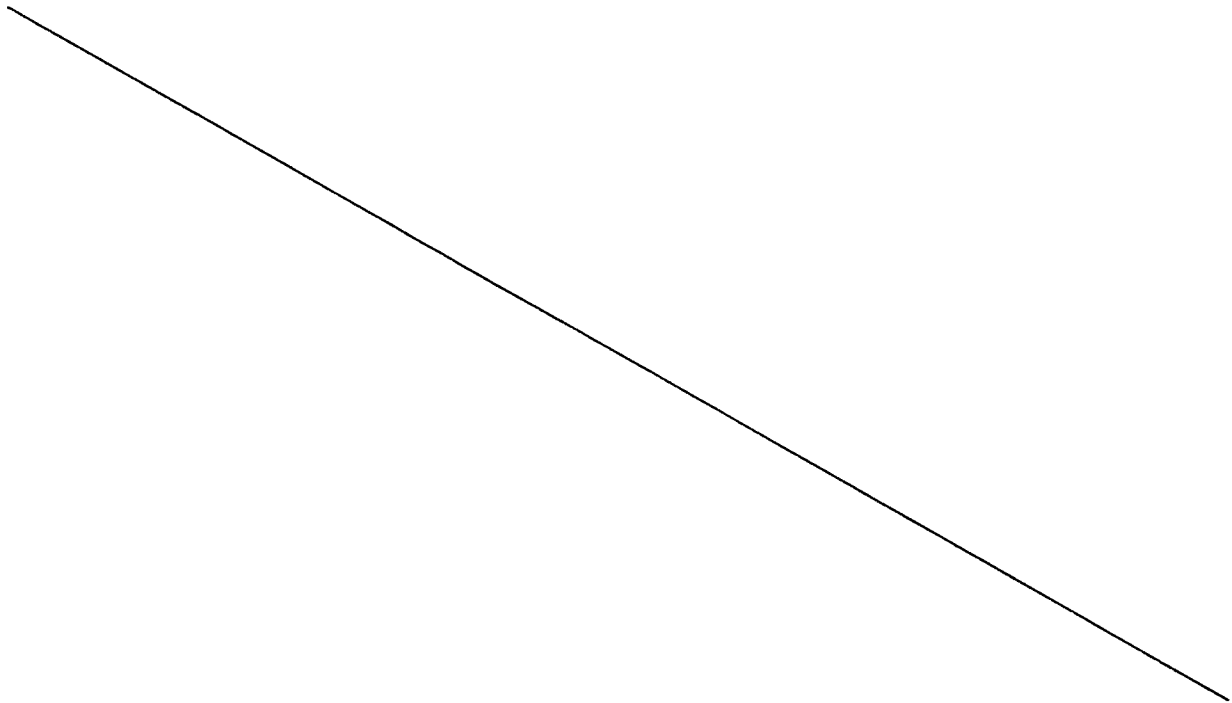
Yarrowia lipolytica is a hemiascomycetous yeast that is readily isolated from various food products. It was originally classified as a *Candida lipolytica* until the perfect form (sexual stage) was identified, and then it was reclassified first as *Endomycopsis lipolytica*, then as *Saccharomycopsis lipolytica* and finally as *Y. lipolytica*. It is not considered to be pathogenic. Intravenous injection into mice was not associated with any mortality and no viable cells could be recovered after 6 days. It is a strict aerobe, unable to survive under anaerobic conditions with a maximum growth temperature of ~32°C (DuPont, 2006).

Y. lipolytica is permitted as secondary direct food additive for fermentation production of food grade citric acid (21 CFR 173.165). It has also been produced commercially as a source of single cell protein for animal feeds (Toprina) and to produce γ -decalactone as a flavoring agent. Other applications include production of wax esters, 2-ketoglutaric, 2-hydroxyglutaric and

isopropylmalic acids and secretion of heterologous proteins, including several food enzymes. *Y. lipolytica* secretes lipases and proteases that contribute to the ripening of dairy products. Its lipase may also be useful for the synthesis of 2,4-dimethylglutaric acid monoesters and transesterification of meso-cyclopentane diols (DuPont, 2006).

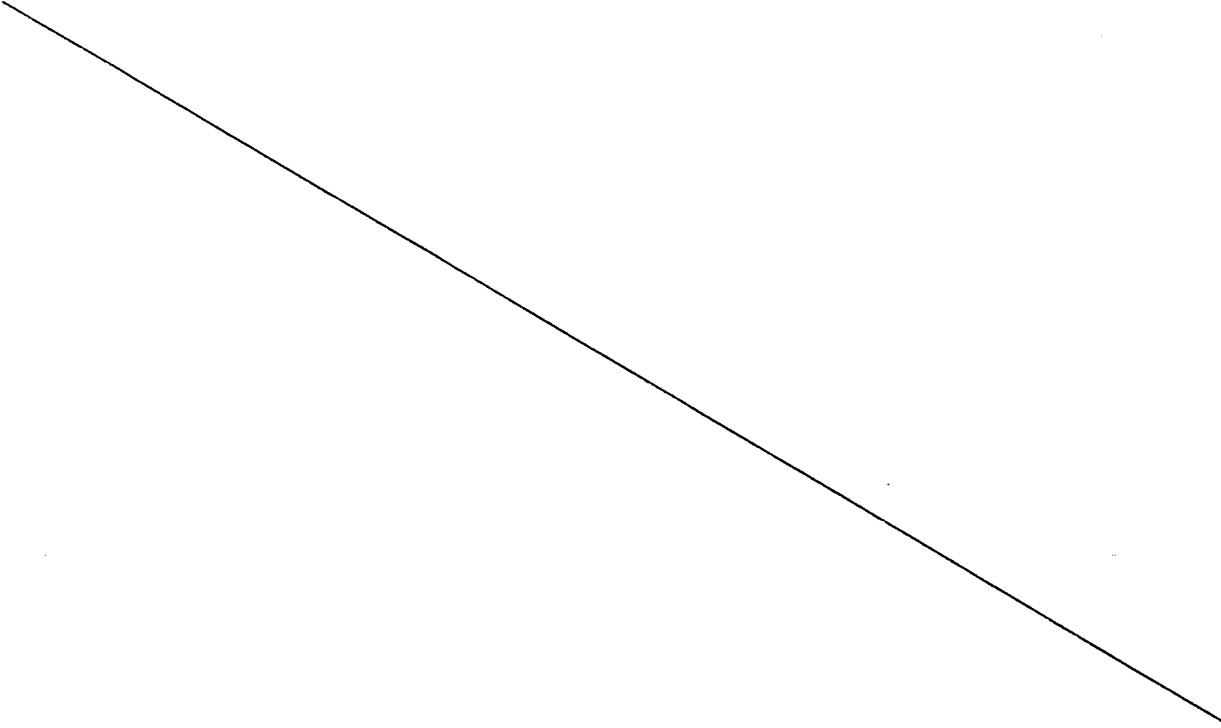
Oleaginous strains can accumulate more than 30% dry cell weight (dcw) as oil when starved for nitrogen in the presence of excess glucose or alkanes. The composition of the oil depends on the carbon source. Oil from glucose-grown cells is comprised mostly of triglycerides and a small amount of sterols. Oleic acid is the prominent fatty acid. Oil from oleic acid-grown cells contains more sterols and oleic acid than glucose-grown cells (DuPont, 2006).

The *Y. lipolytica* genome has been sequenced. It is comprised of 21Mb DNA containing 6650 open reading frames (ORFs) arranged on 6 chromosomes (DuPont, 2006).



Pages 6 through 14

**REDACTED IN ITS ENTIRETY
CONTAINS
TRADE SECRET
CONFIDENTIAL
COMMERCIAL
INFORMATION**



5.0 SAFETY OF THE MICROORGANISM UTILIZED IN THE PRODUCTION OF EPA-RICH TRIGLYCERIDE OIL

Yarrowia lipolytica is a dimorphic hemiascomycetous yeast that is readily isolated from various food products. It was originally classified as a *Candida lipolytica* until the perfect form (sexual stage) was identified, and then it was reclassified first as *Endomycopsis lipolytica*, then as *Saccharomycopsis lipolytica* and finally as *Y. lipolytica*. All strains are heterothallic (mating types MATA and MATB) and both haploid and diploid cells are vegetatively stable. Mating frequencies are very low and dependent on genetic background, growth medium, cell density, growth phase and temperature (DuPont, 2006). *Y. lipolytica* is not considered pathogenic (Holzschu *et al.*, 1979). Intravenous injection into mice was not associated with any mortality and no viable cells could be recovered after 6 days (DuPont, 2006).

Y. lipolytica is a strict aerobe with a maximum growth temperature of approximately 32 °C, and is able to utilize aliphatic carbon sources, including fatty acids and alkanes (DuPont, 2006;

Kerscher *et al.*, 2002; Madzak *et al.*, 2004). It has been used for numerous biotechnological applications. Traditional roles include processing of cheese and other dairy products, but it has also been exploited for the production of single cell protein for animal feeds, for the production of organic compounds like citric acid and α -ketoglutaric acid, and for the bioconversions of aliphatic compounds (Arzumanov *et al.*, 2000; Chernyavskaya *et al.*, 2000; Suzzi *et al.*, 2001; Vannini *et al.*, 2001).

Y. lipolytica differs considerably from other ascomycetous yeasts on the genetic level (DuPont, 2006). Its secretory apparatus (importance of the co-translational pathway, high efficiency, and low overglycosylation) make it closer to mammalian cells than to many other yeasts in some regards, and thus make it a useful host, especially for the production of complex proteins. Vectors and recipient strains are available and easy to handle, and amplification systems and the potential for scaled up production has been studied (Madzak *et al.*, 2004).

Oleaginous strains can accumulate more than 30% dcw as oil, when starved for nitrogen in the presence of excess glucose or alkanes. The composition of the oil depends on the carbon source. Oil from glucose-grown cells is comprised mostly of triglycerides and a small amount of sterols. Oleic acid is the prominent fatty acid. Oils from oleic acid-grown cells contain more sterols and oleic acid than glucose-grown cells (DuPont, 2006).

Y. lipolytica is permitted as secondary direct food additive for fermentation production of food grade citric acid (21 CFR 173.165). It has also been produced commercially as a source of single cell protein for animal feeds (Toprina) and to produce γ -decalactone as a flavoring agent. Other applications include production of wax esters, 2-ketoglutaric, 2-hydroxyglutaric and isopropylmalic acids and secretion of heterologous proteins, including several food enzymes. *Y. lipolytica* secretes lipases and proteases that contribute to the ripening of dairy products. Its lipase may be also useful for the synthesis of 2,4-dimethylglutaric acid monoesters and transesterification of meso-cyclopentane diols (DuPont, 2006).

The *Y. lipolytica* genome has been sequenced. It is comprised of 21Mb DNA containing 6650 ORFs arranged on 6 chromosomes. It is considered to be amenable for genetic development and to be a reliable and versatile host for heterologous production as it combines performance and regularity (Ogrydziak, 1988; Muller *et al.*, 1998; Kerscher *et al.*, 2002; Madzak *et al.*, 2004). As of 2004, forty-two heterologous proteins have been produced using *Y. lipolytica* (Madzak *et al.*, 2004).

6.0 OVERVIEW OF LONG CHAIN POLYUNSATURATED FATTY ACIDS

6.1 Overview

Long chain polyunsaturated fatty acids (LCPUFAs) are fatty acids of 20 carbons or more in length with two or more methylene-interrupted double bonds in the *cis* position. PUFAs can be grouped into two main families, omega-6 (or n-6) or omega-3 (or n-3), depending on the position of the first double bond proximate to the methyl end of the fatty acid. PUFAs are important structural components, modulating membrane fluidity and permeability and serving as precursors for a number of biologically active molecules such as eicosanoids, growth regulators, and hormones (Uauy *et al.*, 1996; Jump, 2002; La Guardia *et al.*, 2006).

Humans lack the enzymes required for the synthesis of the essential fatty acids linoleic acid and α -linolenic acid (Gebauer *et al.*, 2006). However, questions exist whether α -linolenic acid is essential *per se*, or whether eicosapentaenoic acid (EPA), which is derived from α -linolenic acid, and docosahexaenoic acid (DHA), which is in turn produced from EPA, are necessary (Spector, 1999). Thus, linoleic acid and α -linolenic acid must be supplied in the diet (Truska *et al.*, 2006). Although EPA and DHA can be synthesized from ALA, the extent of this metabolic conversion is limited at best. As such, dietary sources rather than metabolic conversion, constitutes the primary source of these fatty acids.

Common omega-6 fatty acids in the human diet are arachidonic acid, present in animal meat, and linoleic acid, present in vegetable oils, seeds, and nuts. Linoleic acid can be converted into arachidonic acid by desaturase enzymes. Major dietary sources of omega-3s include nuts, seeds, and vegetables oils containing α -linolenic acid (ALA), which can be converted to EPA and then DHA. In addition, EPA and DHA are consumed directly, with fatty fish serving as the most significant source. The α -linolenic acid, EPA, and DHA content of select foods is shown in Table 6-1.

Table 6-1 Omega-3 Fatty Acid Content of Select Foods (from DeFilippis and Sperling, 2006).

Food Item	EPA	DHA	ALA
Fish			
Catfish	Trace	0.2	0.1
Cod	Trace	0.1	Trace
Mackerel	0.9	1.4	0.2
Salmon			
Farmed	0.6	1.3	Trace
Wild	0.3	1.1	0.3
Canned	0.9	0.8	Trace
Salmon, Chinook	1.0	0.9	Trace
Swordfish	0.1	0.5	0.2
Tuna, Bluefin	0.3	0.9	-
Tuna, Light			
Canned in oil	Trace	0.1	Trace
Canned in water	Trace	0.2	Trace
Tuna, White			
Canned in oil	Trace	0.2	0.2
Canned in water	0.2	0.6	Trace
Shellfish			
Lobster	-	-	-
Mussels	0.2	0.3	Trace
Shrimp	0.3	0.2	Trace
Nuts and seeds			
Butternuts	-	-	8.7
Flaxseed	-	-	18.1
Walnuts	-	-	9.1
Plant oils			
Canola	-	-	9.3
Flaxseed	-	-	53.3

Measurements are expressed in grams per 100 grams (3.5 oz) of food item.

Trace – 0.1; (-) = 0 or no data

Adapted from the US Department of Health and Human Services

Estimates of dietary intake of omega-3 and omega-6 fatty acids vary within and between different populations, although generally, current intake of PUFAs consists primarily of n-6 fatty acids. The competition for desaturases and elongases in omega-3 and omega-6 PUFA metabolisms results in inverse effects on tissue concentrations of these fatty acids (Cunnane, 2003). The regulation of omega-6 and omega-3 fatty acid metabolism is complex, as EPA, DHA, arachidonic acid, α -linolenic acid, and even linoleic acid itself can inhibit the conversion of linoleic acid to arachidonic acid (Chen and Nilsson, 1993; Emken *et al.*, 1994; 1998; 1999; Sauerwald *et al.*, 1996).

It has been suggested that overall health may be improved by increasing omega-3 polyunsaturated fatty acid (PUFA) intake (Simopoulos, 1991). Estimates of paleolithic human diets suggest that the amounts of omega-3 PUFA and omega-6 PUFA were equal during early human evolution. Changes in food production have greatly altered those proportions in the last 100 to 150 years (Simopoulos, 1991). Intakes of omega-6 PUFAs are now up to 20 to 25 times higher than omega-3 PUFAs in the U.S. (Simopoulos, 1991; DeFilippis and Sperling, 2006). However, based on the limited studies in animals, children, and adults, a reasonable linoleic: α -

linolenic acid ratio of 5:1 to 10:1 has been recommended for adults (FAO/WHO, 1994). Numerous reviews in the literature have reported health benefits associated with even lower ratios.

The mean PUFA intake for men is 20.0 g/day and 16 g/day for women, with LA contributing 17.9 g/day and 13.5 g/day for total PUFA intake in men and women, respectively. The mean EPA + DPA + DHA intake was reported to be 0.17 g/day for men and 0.11 g/day for women. Grain products, meat, poultry, and fish account for most ALA intake (Ervin *et al.*, 2004; Food Surveys Research Group U, 2005). NHANES III reported a median intake of EPA + DHA of 0 and < 1 g/day of ALA. Meat, poultry, and fish contribute all of the EPA and DHA in women's diet and most of the intake in men, where eggs also contribute (Food Surveys Research Group U, 2005).

In the U.S., the Institute of Medicine (IOM), a subdivision of the National Academies of Sciences (NAS), made intake recommendations in its report on macronutrients (IOM, 2005) for omega-3 fatty acids and long-chain omega-3 fatty acids, DHA and eicosapentaenoic acid (EPA). The IOM Report sets an adequate intake (AI) for α -linolenic acid (ALA) of 1.6 g per day for adult men and 1.1 g per day for adult women. The IOM Report further elaborates on the AI for ALA and concludes that, "EPA and DHA can contribute approximately 10 percent of the total omega-3 fatty acid intake and therefore up to this percent can contribute towards the AI for α -linolenic acid". In further recognition of the importance of DHA, the IOM Report concludes that the acceptable macronutrient distribution range (AMDR) for ALA "is set at 0.6 to 1.2 percent of energy" and "up to 10 percent of this range can be consumed as eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA)".

In addition, the IOM panel found that: "[a] growing body of literature suggests that higher intakes of α -linolenic, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) may afford some degree of protection against coronary heart disease (CHD). Because the physiological potency of EPA and DHA is much greater than that for α -linolenic acid, it is not possible to estimate one AMDR for all omega-3 fatty acids. Up to 10 percent of the AMDR can be consumed as EPA and/or DHA".

Gebauer *et al.* (2006) reported that the evidence base supports a dietary recommendation of approximately 500 mg/day of EPA and DHA for cardiovascular disease risk reduction. For treatment of existing cardiovascular disease, 1 g/day is recommended (Gebauer *et al.*, 2006).

Canada was the first country to make separate recommendations for omega-3 and omega-6 fatty acid consumption. Health and Welfare Canada recommended daily intakes of omega-3 PUFA in the 1.0 to 1.6 g range for adults older than 19 years, with an additional 0.05 to 0.16 g for pregnancy, and 0.25 g for lactation. In addition to the recommendations of the IOM and Health and Welfare Canada, many other organizations have now made recommendations for total long-chain omega-3 fatty acid intake. These values are summarized in Table 6-2.

Table 6-2 International Recommendations for long-chain omega-3 fatty acids (From Gebauer *et al.*, 2006).

Source	Date	Total n-3 % of energy	ALA g/day	EPA + DHA mg/day	n-6:n-3
NATO workshop ¹	1989	-	3	800	4:1
UK Committee on Medical Aspects of Food Policy ²	1994	0.2	-	100-200	-
ISSFAL workshop ³	1999	1	2.2	650	-
ANC (France) ⁴	2001	0.8-1	1.8	450 (DHA: 110-120)	5:1
Eurodiet ⁵	2000	-	2	200	-
Health Council of the Netherlands ⁶	2001	1	-	200	7.5:1
American Heart Association ⁷	2002	-	-	~ 1g/d (2 ° prev CHD)	-
US National Academies of Science, Institute of Medicine ⁸	2002	-	1.4	~ 140	-
European Society of Cardiology ⁹	2003	-	-	~ 1g/d (2 ° prev CHD)	-
WHO FAO ¹⁰	2003	1-2	-	400-1000 (12 fish meals/week)	-
ISSFAL ¹¹	2004	-	1.6	≥ 500	-
UK Scientific Advisory Committee on Nutrition ¹²	2004	-	-	Minimum 2 portions fish/week (1 oily); assumes 450 mg	-

Abbreviations: ALA, α -linolenic acid; ANC, Apports Nutritionnelles Conseilles; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAO, Food and Agriculture Organization; ISSFAL, International Society for the Study of Fatty Acids and Lipids; NATO, North Atlantic Treaty Organization; WHO, World Health Organization

References:

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- UK Scientific Advisory Committee on Nutrition. Internet: <http://www.sacn.gov.uk/reports/> (accessed 17 April 2006)

6.1.1 Eicosapentaenoic acid (EPA)

Eicosapentaenoic acid (EPA) is the primary omega-3 fatty acid substrate for *in vivo* synthesis of 3-series prostanoids and especially 5-series leukotrienes. EPA may be derived from direct dietary intake, from desaturation and elongation of α -linolenic acid or from oxidative shortening of DHA.

6.1.2 Docosahexaenoic Acid (DHA)

Docosahexaenoic acid (DHA) is associated with membrane structural phospholipids, particularly those of the cerebral cortex, retina, testis and sperm. Omega-3 fatty acids, and DHA in particular, can increase cell membrane fluidity and deformability such as in erythrocytes (Simopoulos, 1991; Engler *et al.*, 1990). Its role as a nutrient essential to the optimum growth and development of visual and cognitive functions in premature and term infants is now recognized (Salem *et al.*, 1986).

6.2 Absorption, Distribution, Metabolism, and Excretion of Omega-3 Fatty Acids

The absorption, distribution, metabolism, and excretion of omega-3 fatty acids was recently reviewed by the Institute of Medicine. An overview is presented below.

6.2.1 Absorption

The digestion and absorption of omega-3 fatty acids is similar to that of other long-chain fatty acids (IOM, 2005). Following ingestion, triglycerides are generally emulsified in the stomach and duodenum, where they are mixed with bile salts and enzymatically hydrolyzed by pancreatic lipase. Pancreatic lipase has a high degree of specificity for the *sn*-1 and *sn*-3 positions of the triglyceride. The digested triglycerides are generally absorbed by the intestinal mucosa either as free fatty acids or as the *sn*-2 monoglyceride.

EPA and DHA are incorporated into circulating triglycerides in the same proportion that they appear in the dietary oil (Bronsgeest-Schoulte *et al.*, 1981). Their *in vivo* peroxidation is the same as that seen for oleate or linoleate (Higdon *et al.*, 2000). EPA and DHA show specificity for different phospholipids (Mori *et al.*, 1987). Cholesterol esters become enriched with EPA but not with DHA (Holub *et al.*, 1987).

6.2.2 Distribution

The intestinal mucosa cells absorb the free fatty acids and *sn*-2 monoglycerides and convert these components into new triglycerides and phospholipids. Triglycerides accumulate in large

1 mm droplets containing about 88% triglyceride, 8% phospholipids, 3% cholesterol ester, and 1% to 2% protein. These droplets are ejected from gut mucosal cells by exocytosis as chylomicrons. Chylomicrons leave the intestine with lymph and enter the bloodstream via the thoracic duct.

6.2.3 Metabolism

Fatty acids are building blocks for new membranes and provide an energy-rich fuel source for cellular metabolism. In adipose cells, for example, free fatty acids produced by lipoprotein lipase are converted to triglycerides and stored for later use. When fat is needed for fuel, adipose tissue triacylglycerol undergoes lipolysis and free fatty acids are released into the circulation bound to albumin. Fatty acids then undergo β -oxidation to produce 2-carbon substrates from the Krebs's cycle. The Krebs's metabolic cycle produces CO_2 and NADPH. NADPH is then oxidized in mitochondria to produce ATP, releasing two electrons added to O_2 to make H_2O . The ATP produced in this way provides energy for all cellular functions (IOM, 2005).

The biosynthesis of long-chain polyunsaturated fatty acids from essential 18-carbon fatty acid precursors α -linolenic acid and linoleic acid obtained from the diet involves the sequential addition of 2-carbon units and desaturations catalyzed by $\Delta 5$ - and $\Delta 6$ -desaturases. The ability to convert α -linolenic acid to EPA and DHA and the pathways utilized in this process differ among mammalian species (IOM, 2005). This process is discussed in greater detail in Section 5.3.

6.2.4 Excretion

Fatty acids are almost completely absorbed and either oxidized to carbon dioxide and water, incorporated into tissue lipids, or utilized in eicosanoid synthesis. Small amounts of omega-3 fatty acids are lost during sloughing of skin and other epithelial cells.

6.3 Biosynthesis of Omega-3 Fatty Acids

6.3.1 Overview

The liver is the primary organ of lipid metabolism in humans. The first step in the biosynthesis of 20-carbon fatty acids is desaturation of the essential fatty acids linoleic or α -linolenic acid at the $\Delta 6$ position, followed by elongation. These 20-carbon products are desaturated by a $\Delta 5$ desaturase to produce ARA or EPA, respectively. The $\Delta 6$ -desaturation step is rate limiting in this metabolic pathway (Bernert and Sprecher, 1975; Yamazaki *et al.*, 1992; Willis and Browse, 1999).

Conversion of dietary ALA into EPA is limited, and efficacy of LCPUFA synthesis decreases down the cascade of ALA conversion such that DHA synthesis from ALA is even more restricted than that of EPA (Brenna, 2002; Sinclair *et al.*, 2002). Normally, most dietary LA and ALA are β -oxidized to provide energy and only a small portion of them (3.0% and 1.5%, respectively) are converted to longer PUFAs to meet the metabolic needs (Cunnane and Anderson, 1997). PUFA formation can be further hampered by other dietary components, hormonal imbalances, or certain chronic diseases (Huang *et al.*, 2004). However, the synthesis of EPA and DHA from α -linolenic acid is not efficient, particularly when diets are rich in linoleic acid, which competes with α -linolenic acid for elongation and desaturation enzymes. As a result, it is usually necessary to obtain these acids directly from the diet in order to attain nutritionally adequate levels (Green, 2004).

6.3.2 The $\Delta 6$ -Desaturase/Elongation Pathway

Biosynthesis of LCPUFAs from their C18-PUFA precursors occurs *via* the conventional aerobic or $\Delta 6$ -pathway in most LCPUFA-synthesizing eukaryotes. LCPUFA biosynthesis starts with linoleic acid, which is either desaturated at the $\Delta 6$ -position and progresses down the omega-6 pathway, or is desaturated at the $\Delta 15$ -position to produce α -linolenic acid, and progresses down the omega-3 pathway. Subsequent desaturation and elongation steps occur in each pathway, carried out by shared enzymes. Fatty acids are linked either to phosphatidylcholine or coenzyme A. All desaturations occur to fatty acids coupled to phosphatidylcholine while elongations occur to fatty acids coupled to CoA. Enzymes termed acyltransferases are responsible for shuttling fatty acids between the phosphatidylcholine and CoA pools (Robert, 2005).

6.3.2.1 EPA synthesis

The first three steps in the production of EPA involve a $\Delta 6$ -desaturation of α -linolenic acid to SDA, a chain elongation to form omega-3-eicosatrienoic acid (ETA), and a $\Delta 5$ -desaturation to form EPA. Alternate pathways have been identified in other organisms (e.g., some protists, including *Euglena gracilis*) and in some mammalian tissues, namely testis of rats and humans. In this pathway, the essential 18-carbon fatty acids are elongated to 20-carbon chain lengths followed by consecutive $\Delta 8$ - and $\Delta 5$ -desaturations (Huang *et al.*, 2004; Green, 2004; Hulanicka *et al.*, 1964; Albert and Coniglio, 1977; Albert *et al.*, 1979).

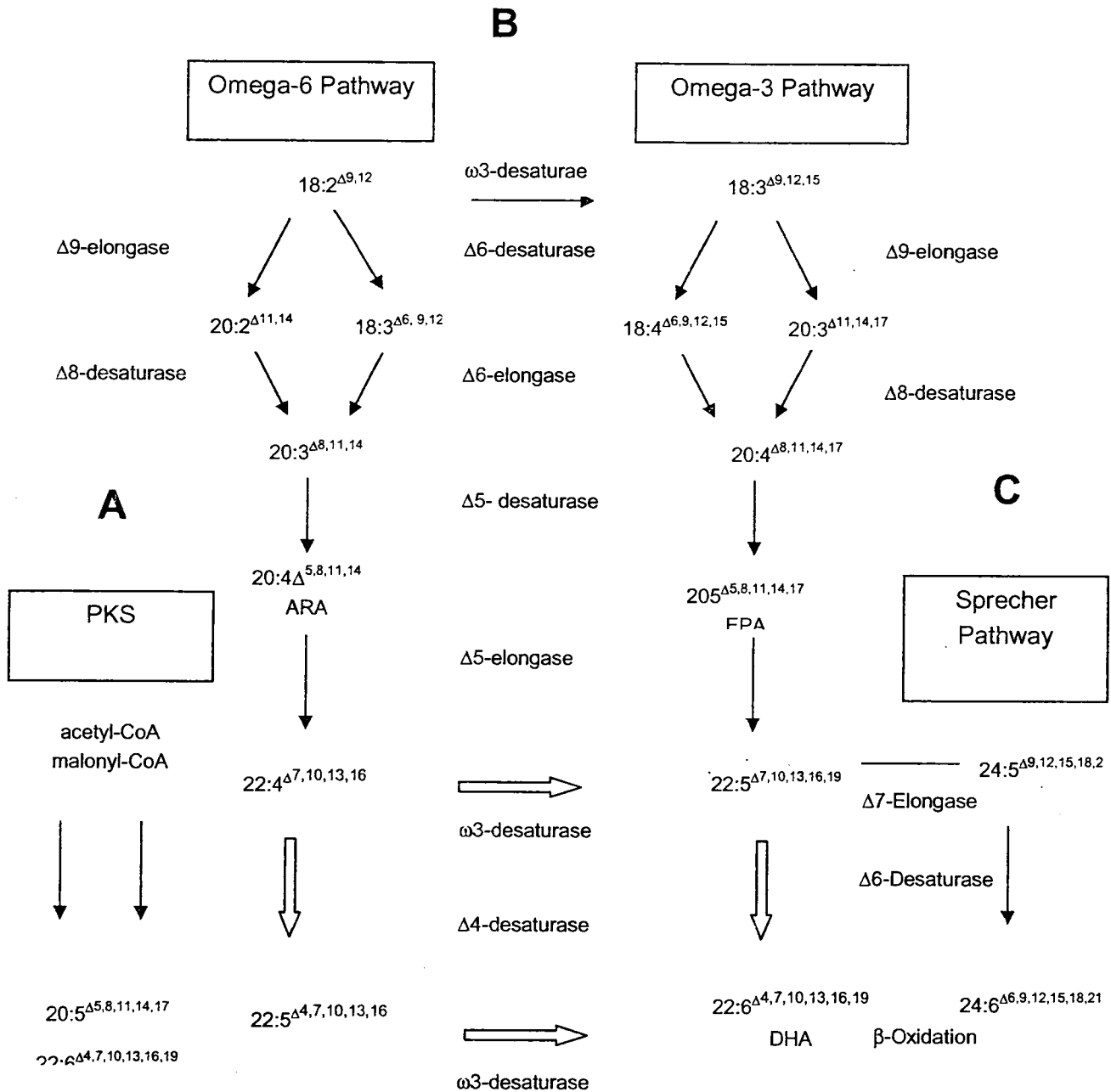
6.3.3 Alternate Pathways

Plants convert oleic acid (18:1) to linoleic acid (18:2) and linolenic acid [18:3(n-3)], however mammals lack the $\Delta 12$ - and omega-3 desaturase activities and obtain linoleic acid and α -linolenic acid from their diets (Bergé and Barnathan, 2005). Other organisms use a pathway mediated by the activities of $\Delta 9$ -elongase and $\Delta 8$ -desaturase. This $\Delta 8$ -desaturase activity has

also been observed in breast cancer and glioma cell lines (Grammatikos *et al.*, 1994; Bardon *et al.*, 1996; Cook *et al.*, 1991). Some marine bacteria and thraustochytrids are able to synthesize eicosapentaenoic acid and docosahexaenoic acid anaerobically by a completely different polyketide-like pathway (Metz *et al.*, 2001) that does not involve desaturation/elongation. Others utilize a group of enzymes designated the omega-3-desaturases that can catalyze the conversion of omega-6 to omega-3 (e.g., linoleic acid to α -linolenic acid, arachidonic acid to EPA). This pathway occurs frequently in lower organisms including algae, fungi, mosses, and worms, however, mammals lack both the omega-6- and the omega-3-desaturases (Meyer *et al.*, 2003).

An overview of the various pathways leading to EPA and DHA in microorganisms, plants, and animals is shown in Figure 6-1.

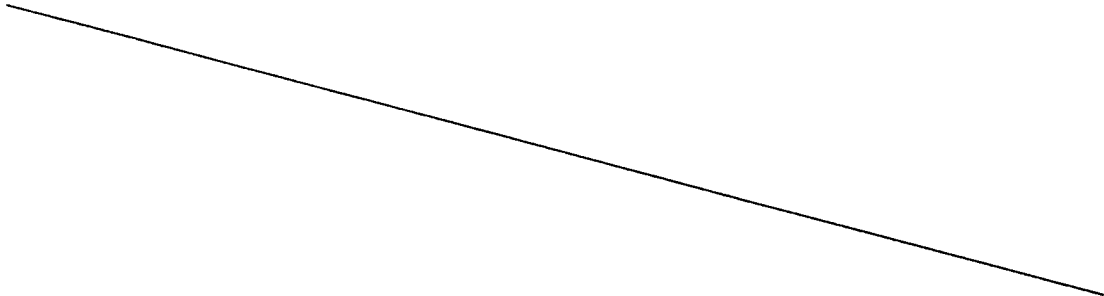
Figure 6-1 Alternative routes leading to EPA and DHA in microorganisms, plants, and animals (adapted from Meyer *et al.*, 2003)



(A) Polyketide synthase (PKS) systems producing either EPA or DHA (in marine bacteria (and possibly *Schizochytrium*)). (B) The omega-3- and omega-6 pathways of LCPUFA biosynthesis, including the routes that use $\Delta 9$ -elongase and $\Delta 8$ -desaturase. (C) The Sprecher Pathway operating in mammals. The omega-3-desaturase(s) may accept further omega-6 substrates in addition to the arrows included in the figure. It should be noted that switching from the omega-6 to the omega-3 track is not a universal alternative. It occurs frequently in lower organisms (algae, fungi, mosses, worms), but not in mammals, which lack both the omega-6 and the omega-3-desaturases.

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6.3.5 Dietary Effects of Supplementary Omega-3 Fatty Acids

Arterburn *et al.* (2006) reviewed the tissue distribution, interconversion, and dose response data on omega-3 fatty acids. The available data suggests that supplementation of the typical Western diet with even large doses of ALA results in only marginal changes in ALA plasma concentrations, small increases in plasma EPA, and no effects on plasma DHA. These data are consistent with the high oxidation rate and low fractional conversion rates for the n-6 fatty acids known to occur in humans.

EPA concentrations in plasma increase in response to dietary EPA, but DHA levels remain unaffected. Dietary DHA results in a dose-dependent, saturable (at approximately 2 g/day) increase in plasma DHA concentrations and modest increases in EPA concentrations. Plasma DHA concentrations equilibrate in approximately 1 month and then remain at steady state throughout supplementation. Both dietary DHA and EPA reduce plasma arachidonic acid concentrations. Tissue concentrations of DHA and EPA can also be elevated through dietary supplementation. Only minute quantities of EPA are generally present in tissues. DHA concentrations range from 5 to 30-fold in most organs, and several hundred-fold more in brain and retina.

As reviewed in Arterburn *et al.* (2006), EPA is more rapidly cleared from plasma than DHA. Plasma EPA concentrations decreased rapidly after cessation of omega-3 supplementation, and approached baseline levels within 4 weeks, whereas DHA concentrations remained above baseline levels after 24 weeks. Both DHA and EPA decrease more rapidly in plasma cholesterol esters than in phospholipids. EPA washout from red blood cells is also faster than that of DHA. Whereas DHA accumulates predominantly in phospholipids, EPA is more equally distributed between neutral lipids (sterol esters and triacylglycerols) and phospholipids. This differential distribution may be linked to differences in the kinetics of washout and their saturation dynamics in plasma and availability to tissues.

Arterburn *et al.* (2006) suggested that the most predictable way to increase a specific long-chain omega-3 fatty acid in plasma, tissues, or human milk is to supplement with the fatty acid of interest.

6.4 Physiological Role of Omega-3 Fatty Acids

As noted in Section 6.1, polyunsaturated fatty acids are important structural components, modulating membrane fluidity and permeability. Docosahexaenoic acid (DHA) is associated with membrane structural phospholipids, particularly those of the cerebral cortex, retina, testis and sperm, and as such, inadequate intakes of omega-3 fatty acids are associated with impaired visual and neural function, as well as a number of other impaired health endpoints relating to the effects of omega-3 fatty acids on various physiological processes. There is no accepted cut-off concentration of plasma or tissue concentrations below which these functions are impaired (IOM, 2005).

In addition to structural roles, omega-6 and omega-3 fatty acids serve as precursors to two distinct classes of eicosanoid metabolites whose hormone-like properties play critical roles in modulating the response of several homeostatic and immune mechanisms. The omega-6 acid arachidonic acid (ARA) is the precursor of the 2-series of cyclooxygenase generated prostanoids (prostaglandins and thromboxanes) and the 4-series of lipoxygenase-generated leukotrienes, while the omega-3 fatty acid EPA is a precursor of the 3-series prostanoids and the 5-series leukotrienes. Corresponding members of the two series often have differing or even opposing activity; with the ARA series generally activating immune and inflammatory processes, the DHA/EPA series generally decreasing them (Simopoulos, 1991). The relative balance among members of these two eicosinoid families is important in determining their net local effect. As discussed in Section 5.3, the synthesis of omega-6 and omega-3 PUFAs is mediated by the same enzymes, which generally appear to have no particular preference for substrate. Thus, the balance of intake of omega-6 and omega-3 fatty acids will determine the types and amounts of eicosanoids in the body (Sayanova and Napier, 2004).

Eicosanoids derived from omega-6s are generally pro-inflammatory and pro-aggregatory, whereas those from omega-3s are predominately anti-inflammatory and inhibit platelet aggregation (Simopoulos, 1999). This may account for the cardioprotective effects of omega-3's (DeFilippis and Sperling, 2006). In addition to markers of vascular inflammation, omega-3s may beneficially influence other factors related to cardiovascular disease risk, including ventricular arrhythmia's, thrombosis, triglycerides, apolipoprotein B, high-density lipoprotein, adhesion molecule expression in plaque, platelet-derived growth factor, nitric oxide induced endothelial relaxation, and blood pressure (Kris-Etherton *et al.*, 2003; Simopoulos, 1999; Weber and Leaf, 1991; Weber, 1989; Harper and Jacobson, 2001).

Competition occurs between ARA and EPA (but probably not DHA) for prostaglandin and leukotriene synthesis at the cyclooxygenase and lipoxygenase level, EPA being a better substrate for lipoxygenase than for cyclooxygenase (Linscheer and Vergroesen, 1994). Ingested and derived DHA and EPA may partially replace ARA in cell membranes, especially those of platelets, erythrocytes, monocytes, neutrophils and liver cells (Simopoulos, 1991). In the case of DHA, competition with ARA would be expected to arise in two ways. Partial

replacement of ARA by DHA in membrane lipids may initially increase and then subsequently decrease the amount of ARA substrate available for prostanoid and leukotriene synthesis. Since the availability of dietary and derived ARA is comparatively high, competition at this level seems unlikely to assume significant proportions unless DHA intake is elevated substantially. The second avenue is *via* conversion of DHA to EPA [(Brossard, *et al.*, 1996) which in turn may directly compete with ARA for access to synthetic enzymes as stated earlier. Since EPA is a preferred substrate for these enzymes compared to ARA (Poisson *et al.*, 1992; Simopoulos, 1991; Linscheer and Vergroesen, 1994) competition at this level may be significant at relatively low EPA levels.

Accordingly, increased ingestion of omega-3 fatty acids may lead to:

1. decreased production of prostaglandin E₂ (PGE₂) metabolites;
2. decreased production of thromboxane A₂, which is a potent platelet aggregation and vasoconstriction agent;
3. decreased formation of leukotriene B₄, a potent leukocyte chemotaxis and adherence agent and inflammation factor;
4. increased formation of thromboxane A₃, which exhibits only weak platelet aggregation and vasoconstriction activity;
5. an increase in formation of prostacyclin E₃ (PGI₃) without a decrease in PGI₂ leading to an overall increase in prostacyclins which are vasodilators and inhibit platelet aggregation; and
6. increased formation of leukotriene B₅, a weak chemotactic and weak inflammatory agent.

6.4.1 Effects of Omega-3 Fatty Acid Supplementation on Cardiovascular Health

Epidemiological data and prospective randomized trials demonstrate omega-3 fatty acids offer protection against cardiovascular disease (DeFilippis and Sperling, 2006). It is believed these effects are mediated through production of eicosanoids, a class of bioactive molecules derived from omega-3s and omega-6s that include leukotrienes, prostaglandins, and thromboxanes. Thus, dietary intake of these fatty acids may also prove to be useful in moderating the risk of cardiovascular disease. In 2005, the Institute of Medicine (IOM) published *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids*, which contains an extensive review of the epidemiological and clinical evidence on the health benefits associated with omega-3 fatty acids (IOM, 2005). This report summarizes the data on the health benefits associated with diets containing omega-3 fatty acids and goes on to

state that a “growing body of literature suggests that diets higher in α -linolenic acid, EPA, and DHA may afford some degree of protection against CHD”.

Wang *et al.* (2006) recently reported the results of an evidence-based, systematic review of the literature on the effects of omega-3 fatty acids consumed as fish, or fish oils rich in EPA and DHA, or as alpha-linolenic acid, on cardiovascular disease outcomes and adverse effects associated with their consumption. Randomized controlled trials and prospective cohort studies that followed patients for at least 1 year and case control studies that reported intakes of omega-3 fatty acids or fish were included in the review. Evidence suggested that the increased consumption of omega-3 fatty acids from fish or fish oil supplements, but not from alpha-linolenic acid, reduces the rates of all-cause mortality, cardiac and sudden death, and possibly stroke, with stronger evidence of beneficial effects in secondary-prevention settings (patients with a history of cardiovascular disease) than in primary prevention settings (general population with no history of cardiovascular disease).

The most compelling evidence for the cardiovascular benefit provided by omega-3 fatty acids comes from 3 large controlled trials of 32,000 participants randomized to receive omega-3 fatty acid supplements containing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) or to act as controls. These trials showed reductions in cardiovascular events of 19% to 45%. For example, the Japan EPA Lipid Intervention Study (JELIS), 18,645 patients with hypercholesterolemia (70% women; mean age, 61 years) were randomly assigned to either statin alone or statin and pure EPA (1.8 g/d). During the 5-year study, EPA reduced major adverse CV events by 19%. Additionally, the JELIS trial established the safety and efficacy of combination therapy with EPA and a statin vs statin therapy alone for improving CV prognosis (Yokoyama *et al.*, 2007).

Balk *et al.* (2007) published the results of an evidence-based systematic review of the data related to the use of omega-3 fatty acids in a variety of clinical conditions. The authors reported that adequate evidence is available to attribute a health benefit to consumption of supplemental fish oil or dietary fish only with respect to a reduction in cardiovascular events, principally as secondary prevention. Furthermore, fish oils reduce serum triacylglycerol concentrations, reduce blood pressure by a small but significant amount, reduce corticosteroid use in patients with rheumatoid arthritis, and reduce the risk of non-Alzheimer dementia in certain persons and at various doses.

6.5 Safety of Omega-3 Fatty Acids

6.5.1 Overview

The safety of omega-3 fatty acids is supported by their long history of ingestion as a component of the human diet. Although the high LCPUFA doses used in animal studies (>1g/kg body

weight/day) may produce adaptive non-specific effects in liver metabolism and histomorphology, these effects are related to the extra metabolic workload and are not relevant to exposure at much lower doses.

Adverse effects associated with omega-3 polyunsaturated fat consumption have been reported in a few special subpopulations (IOM, 2005). It has been suggested that individuals exhibiting glucose intolerance or diabetic conditions use caution with omega-3 fatty acids as some have required increased doses of hypoglycemic agents (Glauber *et al.*, 1988; Kasim *et al.*, 1988; Friday *et al.*, 1989; Stacpoole *et al.*, 1989; Zambon *et al.*, 1992). Clarke *et al.* (1990) reported increased episodes of nosebleeds in individuals with familial hypercholesterolemia during fish oil supplementation. High levels of eicosapentaenoic acid (EPA) content may reduce growth in infants (Carlson *et al.*, 1993).

The FDA critically evaluated available information on the safety on the omega-3 fatty acids, EPA and DHA, and identified three potential safety issues, namely prolongation of bleeding time, modification of glycemic control, and elevation of LDL cholesterol (US FDA, 1993). Similarly, the Societa Italiana di Nutrizione Umana (1996) and Hughes and Pinder (1997) reported that the intake of excessive omega-3 fatty acids may result in toxic effects, including increased bleeding rate, alteration of immune function, and formation of toxic lipoperoxides (Societa Italiana di Nutrizione Umana, 1996; Hughes and Pinder, 1997). It has been noted that the intake of omega-3 fatty acids should also include adequate quantity of vitamin E or tocopherols to limit oxidation of PUFAs by free radicals (Witting and Lee, 1975; Brude *et al.*, 1997; La Guardia *et al.*, 2006).

A more comprehensive discussion of the potential safety issues associated with omega-3 fatty acids is provided in Sections 6.5.2 through 6.5.5.

The results of numerous clinical studies published since that time indicate the DHA provided in fish or marine-derived oils at levels up to 6 g DHA/person/day would not be expected to produce adverse effects on these parameters (Kroes *et al.*, 2003). These results are consistent with the FDA conclusion in its 1997 menhaden oil decision that the combined consumption of up to 3 g of DHA + EPA/person/day poses no significant risk for bleeding time, produces no clinically significant effect on glycemic control, and is safe with respect to the effect on LDL cholesterol (FDA, 1997).

The US Department of Health and Human Services Agency for Healthcare Research and Quality (2004) identified 148 studies on omega-3 fatty acids that evaluated over 20,000 subjects for adverse effects. The most common side effects were gastrointestinal complaint, reported among 6.6% of patients taking omega-3s versus 4.3% in placebo groups. An increased incidence of bleeding was not observed, and only 1 of the 148 studies reported such an association in patients randomized to 6 g/day of omega-3. There were no reported deaths or life-threatening illnesses as a consequence of omega-3 consumption. No adverse effects were

reported in 77 of the 148 studies. Based on this review, the agency concluded that adverse effects related to consumption of fish oil or α -linolenic supplements appear to be minor.

Wang *et al.* (2006) recently reported the results of an evidence-based, systematic review of the literature on the effects of omega-3 fatty acids consumed as fish, or fish oils rich in EPA and DHA, or as alpha-linolenic acid, on cardiovascular disease outcomes and adverse effects associated with their consumption. Adverse effects were generally considered to be minor. Gastrointestinal side effects were the most often reported, but only 4% of complaints occurred at dosages of less than 3 g/day. GI symptoms increased to approximately 20% at a dose of 4 g/day. Nine studies included in the review addressed the risk of clinically significant bleeding episodes. Four of these studies reported no bleeding in either arm, while in the remaining five, no consistent association found between the omega-3 fatty acid dose and the risk of bleeding, even in those patients taking aspirin or warfarin.

Similarly, in their evidence-based systematic review of the data related to the use of omega-3 fatty acids, Balk *et al.* (2007) found that although only a limited number of the reviewed articles provided information on adverse events, and the reporting of adverse events in these studies was generally inadequate and incomplete (e.g., rates of adverse events were not systematically or consistently monitored and reported), no evidence of substantial adverse events related to omega-3 fatty acid consumption beyond mild gastrointestinal symptoms was found.

6.5.2 Effects on Bleeding Time

As reviewed in Section 6.4, a link between omega-3 polyunsaturated fatty acid intake and reduced risk of coronary heart disease has been suggested, with reduced platelet aggregation proposed as a possible mechanism. This effect may also result in prolonged bleeding time, although intervention studies examining the effects of omega-3 fatty acids on bleeding time have produced equivocal results (IOM, 2005).

Thorngren and Gustafson (1981) reported that ingestion of omega-3 fatty acids by individuals taking anticoagulants such as aspirin, warfarin, and coumadin may result in prolonged bleeding times. Collective analysis of numerous clinical studies with EPA/DHA supplements, ranging in dose levels from 2 to 15 g/day, has shown no dose response for EPA and DHA on bleeding times. None of the studies indicated excessive bleeding times, bleeding episodes, or bruising (IOM, 2005). Some studies have indicated an increased risk of hemorrhagic stroke among Greenland Eskimos with high EPA and DHA intakes (Kromann and Green, 1980; Kristensen, 1983; Pedersen *et al.*, 1999). While a prospective study in the U.S. showed no association between intake of ω -3 fatty acids and risk of hemorrhagic stroke, the median intakes ranged from only 0.077 to 0.481 g/day, which were considered to be relatively low levels of intake in the U.S. (Iso *et al.*, 2001; IOM, 2002). Increased lipid peroxidation and oxidative damage of erythrocytes, liver, and kidney membranes and bone marrow DNA have been reported in feeding studies with DHA in laboratory animals (Ando *et al.*, 1998; Yasuda *et al.*, 1999; Song

and Miyazawa, 2001; Umegaki *et al.*, 2001); however, consumption of vitamin E reportedly reduced or prevented the oxidative damage (Leibovitz *et al.*, 1990; Ando *et al.*, 1998; Yasuda *et al.*, 1999).

Bays (2007) reported that the clinical trials have shown high-dose fish oil omega-3 fatty acid consumption to be safe, even when concurrently administered with other agents that may increase bleeding, such as aspirin and warfarin. He noted that in certain clinical settings, it could be argued that the reported antiatherothrombotic effects of fish oils rich in omega-3 fatty acids are a benefit that outweighs the unproved bleeding risks, especially in select patients at high risk for thrombosis, which may include patients with acute atherosclerotic CAD.

Likewise, a recent comprehensive report by Harris (2007) concluded that virtually no increased risk for clinically remarkable bleeding has been seen in patients with doses of omega-3 fatty acids up to 7 g/d of DHA and EPA,.

6.5.3 Effects on LDL cholesterol

Prospective cohort studies and a trial of secondary prevention of ischemic heart disease suggested that intakes of greater than 1 g/day of omega-3 LCPUFAs offer protection from fatal ischemic heart disease (Hu *et al.*, 2002; Albert *et al.*, 1998, Anonymous, 1999). Likewise, a mixture of EPA and DHA has been shown to lower serum triacylglycerol concentrations, though a slight and, concurrent increase in LDL cholesterol concentrations may occur, particularly in hypertriacylglycerolemic subjects (Harris, 1989; 2003; Davidson *et al.*, 1997; Anonymous, 1999); Mori *et al.*, 2000; Montori *et al.*, 2000; Theobald *et al.* 2004) while favorable effects on lipoprotein particle distributions may occur (Li *et al.*, 2004). Increased LDL levels are associated with increased risk of cardiovascular disease, thus increases in LDL could counteract other beneficial cardiovascular effects associated with omega-3 oils.

Studies using purified EPA or EPA-rich oil generally have not reported increases in LDL cholesterol (Harris, 1997). DuPont's EPA-rich triglyceride oil would thus be anticipated to offer the beneficial health effects associated with omega-3 fatty acids without the potential LDL-increasing effects attributed to DHA.

A decrease in total VLDL particle concentrations has previously been reported after consumption of EPA and DHA-rich fish oil (Li *et al.*, 2004). In the 2005 study by Goyens and Mensink, however, the proportion of EPA in plasma phospholipids was significantly increased with the consumption of both experimental diets, whereas DHA was not affected. This suggested to authors that EPA may be responsible for the changes reported in VLDL concentrations after consumption of fish oil.

6.5.4 Effects on Glycemic Control

Montori *et al.* (2000) reviewed the data from eighteen trials including 823 type 2 diabetic subjects dosed with 3 to 18 g of fish oil per day for 12 weeks. Researchers found that fish oil supplementation in type 2 diabetics lowered triglycerides, raised LDL cholesterol, and had no statistically significant effect on fasting glucose or glycemic control.

6.5.5 Alteration of Immune Function

Suppression of various aspects of human immune function has been reported *in vitro* or *ex vivo* in peripheral blood mononuclear cells, in isolated neutrophils or monocytes, or in subjects provided omega-3 polyunsaturated fatty acids as a supplement or in experimental diets. The minimum dose observed for such an effect was 0.9 g/day EPA and 0.6 g/day DHA given as fish oil for 6 to 8 weeks to healthy adults (Cooper *et al.*, 1993). Single treatment studies suggest that intakes of EPA and DHA at levels 7 to 15 times the typical U.S. intake diminish the potential of the immune system to attack pathogens (Lee *et al.*, 1985; Schmidt *et al.*, 1989; Kelley *et al.*, 1998, 1999). These doses have also reportedly been associated with suppression of inflammatory responses. However, Blok *et al.* (1997) administered fish oil supplements (0, 3, 6, or 9 g/day fish oil supplement resulting in EPA intakes of 0, 0.81, 1.62, or 2.43 g/day and DHA intakes of 0, 0.16, 0.33, or 0.49 g/day) for 1 year to 58 healthy men. *Ex vivo* endotoxin stimulated production of interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , or IL-1Ra did not differ among treatment groups up to 6 months after supplementation was stopped, supporting a lack of long-term adverse effects of fish oil supplementation on cytokine activity.

Hawkes *et al.* (2002) examined the effects of supplementation with DHA-rich tuna oil on the human milk cytokines interleukin 1 β , interleukin 6, and tumour necrosis factor α . DHA levels in maternal plasma, human peripheral blood mononuclear cells (PBMCs), milk, and human milk cells (HMCs) were increased in a double-blind, prospective, randomized trial in which mothers of healthy full-term infants were asked to consume a nutritional supplement containing either 300 mg DHA + 70 mg EPA or 600 mg DHA + 140 mg EPA for 4 weeks. DHA concentrations reportedly increased in a linear manner. Despite the increased n-PUFA concentrations in relevant tissues, no perturbations in cytokine concentrations were observed. Side effects were reported by 7 to 15% of the subjects and included gas (n=4), fishy burps (n=4), unsettled babies (n=1), and rash (n=1).

The effects of dietary fatty acid supplementation from various sources on endothelial function and tone in healthy subjects were examined by Khan *et al.* (2003). Volunteers took oil supplements containing oleic acid rich sunflower oil, evening primrose oil, soybean oil, tuna fish oil (6% EPA acid and 27% DHA), or tuna/evening primrose oil mix for 8 months. Endothelium-dependent and endothelium-independent responses to iontophoresis of acetylcholine and sodium nitroprusside, respectively, were measured in the forearm skin using laser Doppler imaging. Acetylcholine responses were significantly improved after tuna oil supplementation

while significant positive correlations were also seen between acetylcholine responses and omega-3 fatty acid levels in the plasma and erythrocyte membrane phospholipids in subjects consuming the tuna oil supplements. No significant differences were observed with any of the other oils. Total cholesterol, red and white blood cell counts, hemoglobin, and hematocrit levels were not significantly affected by tuna oil supplementation. The authors concluded that modification of the diet involving a 6% increase in EPA acid and 27% DHA might have significant beneficial effects on cardiovascular function and health.

6.5.6 Dosing Recommendations:

Mozaffarian and Rimm (2006) reviewed the available scientific data on the cardiovascular and neurological health benefits of omega-3 fatty acid intake from fish or fish oil, as well as the possible adverse effects associated with environmental contaminants (i.e., mercury, dioxins, and polychlorinated biphenyls) associated with these commodities. Analysis of the data suggested that consumption of 1 to 2 servings of fish per week, particularly oily species higher in EPA and DHA, was associated with a reduced risk of coronary death (36% reduction, 95% confidence interval, 20%-50%; $p < .001$) and mortality (17% reduction, 95% confidence interval, 0%-32%; $p = .046$). Other clinical outcomes may also be favorably affected. For example, there was moderate evidence suggesting a reduced risk of ischemic stroke, and equivocal evidence that fish and fish oils produce modest benefits related to nonfatal myocardial infarctions, progression of atherosclerosis, postangioplasty restenosis, and recurrent ventricular tachyarrhythmias. Limited evidence suggests decreased risk of atrial fibrillation and congestive heart failure associated with intake of fish and fish oils. The data indicated that EPA and DHA intakes of 250 mg/day appear sufficient for primary prevention, with little additional risk reduction at higher intakes (Mozaffarian and Rimm, 2006). For individuals with coronary heart disease, 500 to 1000 mg/day of EPA and DHA is currently recommended to reduce CHD mortality (Kris-Etherton *et al*, 2002; Van de Werf, 2003; Mozaffarian and Rimm, 2006)

It is the position of the American Heart Association that “omega-3 fatty acids benefit the hearts of healthy people, and those at high risk of, or who have cardiovascular disease.” (American Heart Association, 2006)

The American Heart Association recommends patients without documented coronary heart disease consume a variety of fatty fish at least twice per week. Oils and foods rich in alpha-linolenic acid (flaxseed, canola and soybean oils; flaxseed and walnuts) should also be included, although the AHA notes that the extent of *in vivo* conversion to omega 3 fatty acids is small and that more studies are needed to show the beneficial effects of alpha-linolenic acid and heart disease. Patients with documented coronary heart disease are advised to consume about 1 g of EPA + DHA per day, preferably from fatty fish. Capsule form could be considered after consulting with a physician. Similarly, it is recommended that patients who need to lower triglycerides consume 2 to 4 g EPA + DHA per day provided as capsules under a physician's

care. However, as noted by Lee et al. (2008), the lower end of this dose range is ineffective in most patients, and thus clinicians should target between 3.0 and 4.0 g/d to achieve meaningful triglyceride lowering.

The AHA notes that patients taking more than 3 grams of omega-3 fatty acids from capsules should do so only under a physician's care since high intakes could cause excessive bleeding in some people. The AHA noted that increasing omega-3 fatty acid intake through foods is preferable, but that coronary artery disease patients may not be able to get enough omega-3 by diet alone. Likewise, people with high triglycerides, who need even larger doses, can also benefit from supplements. The availability of high-quality omega-3 fatty acid supplements, free of contaminants, is an important prerequisite to their use (American Heart Association, 2006).

A summary of these dosing recommendations is shown in Table 6-2.

Table 6-2 Summary of Omega-3 Dosing Recommendations

Recommending Organization/Researcher	Target Population	Amount Recommended	Suggested Benefit
Mozaffarian and Rimm, 2006	Patients without documented coronary heart disease (CHD)	250 mg EPA +DHA/day	Primary prevention of coronary heart disease(CHD)-related death
American Heart Association	Patients without documented coronary heart disease (CHD)	Eat a variety of (preferably fatty) fish at least twice a week. Include oils and foods rich in alpha-linolenic acid (flaxseed, canola and soybean oils; flaxseed and walnuts).	Omega-3 fatty acids reduce CVD risk through • decreased risk of arrhythmias, which can lead to sudden cardiac death • decreased triglyceride levels • decreased growth rate of atherosclerotic plaque • lower blood pressure (slightly)
Kris-Etherton <i>et al</i> , 2002; Van de Werf, 2003; Mozaffarian and Rimm, 2006	Individuals with coronary heart disease	500 to 1000 mg/day of EPA and DHA	Reduced CHD mortality
American Heart Association	Patients with documented CHD	Consume about 1 g of EPA+DHA per day, preferably from fatty fish. EPA+DHA in capsule form could be considered in consultation with the physician.	Omega-3 fatty acids reduce CVD risk through • decreased risk of arrhythmias, which can lead to sudden cardiac death • decreased triglyceride levels • decreased growth rate of atherosclerotic plaque • lower blood pressure (slightly)
American Heart Association	Patients who need to lower triglycerides	2 to 4 grams of EPA+DHA per day provided as capsules under a physician's care.	Omega-3 fatty acids reduce CVD risk through • decreased risk of arrhythmias, which can lead to sudden cardiac death • decreased triglyceride levels • decreased growth rate of atherosclerotic plaque • lower blood pressure (slightly)

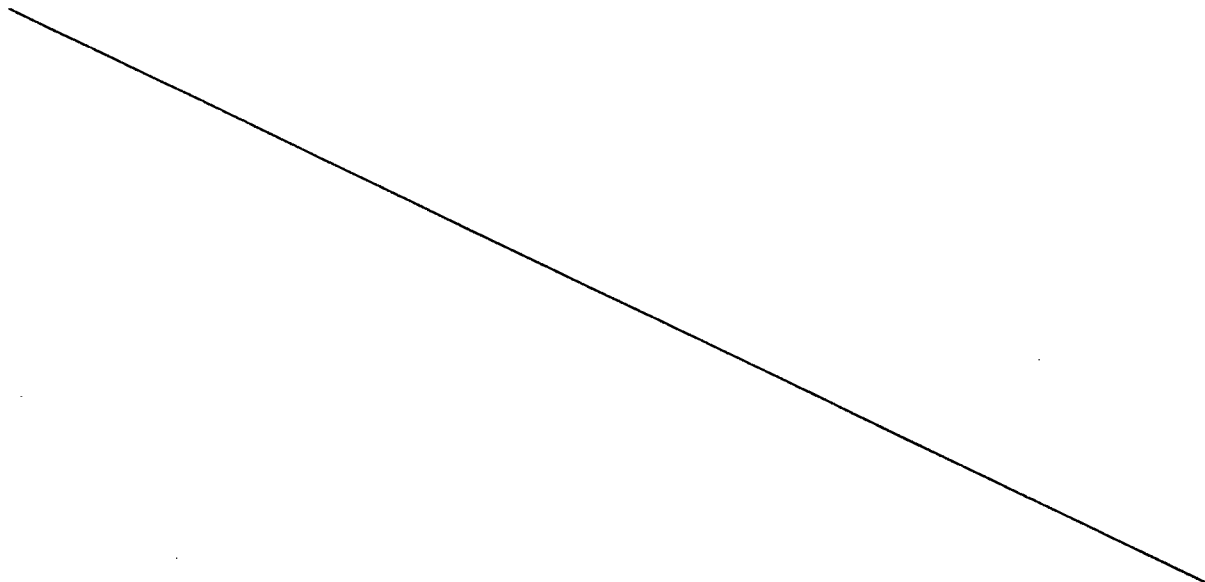
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8.0 SUMMARY AND CONCLUSION

The information presented herein shows that:

- (i) The chemical composition of DuPont's EPA-rich triglyceride oil is well characterized, and the manufacturing process yields a product demonstrated to reproducibly meet compositional specifications.



- (iv) Omega-3 fatty acids, including EPA, have established physiological roles, and reports in the published scientific literature suggest that higher intakes of these fatty acids may afford health benefits

Based on the evidence provided above, including results of preclinical safety studies conducted with DuPont's EPA-rich triglyceride oil, the presence of an approximately 30-fold margin of exposure that exists between the NOAEL from the 28-day repeated dose toxicity study (976 mg/kg/day of EPA) and the maximum recommended dose to consumers (2 g EPA/day, or 33 mg/kg b.w./day for a 60 kg individual), and vast clinical experience with omega-3 fatty acids, including EPA, DuPont concludes that the chronic use of EPA-rich triglyceride oil in dietary supplements at a level of up to 2 g EPA/day will be reasonably expected to be safe.



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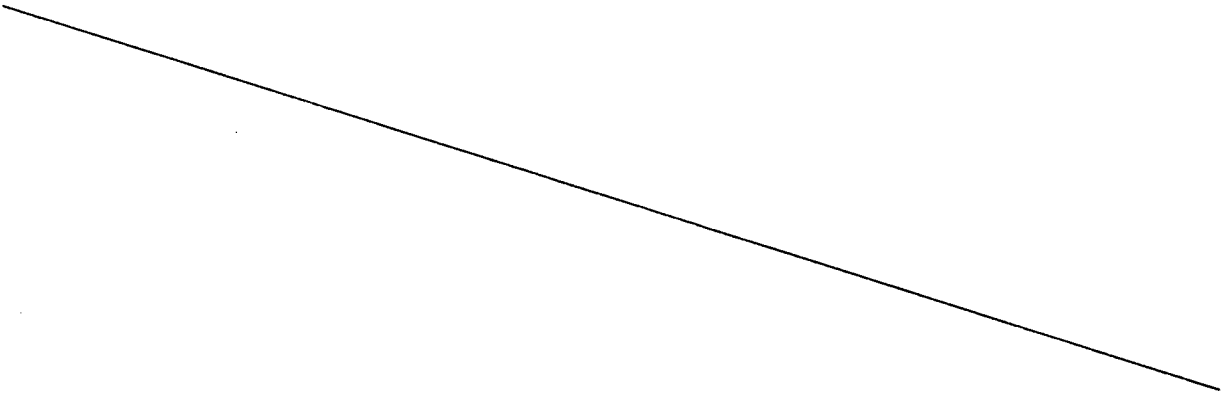
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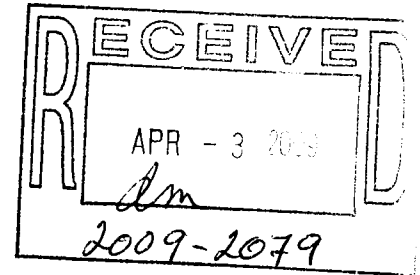
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March 31, 2009

Dr. Vasilios H. Frankos
Director
Division of Dietary Supplement Programs
Office of Nutritional Products, Labeling,
and Dietary Supplements
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740-3835



RE: New Dietary Ingredient Notification for DuPont's EPA-rich triglyceride oil

Dear Dr. Frankos:

We respectfully submit the attached information pursuant to Section 413(a) of the Federal Food, Drug, and Cosmetic Act, in support of DuPont's marketing of the new dietary ingredient EPA-rich triglyceride oil. Three copies of this submission are enclosed.

Based on the data summarized herein, DuPont affirms that a dietary supplement containing EPA-rich triglyceride oil is reasonably expected to be safe when used according to the conditions of use recommended or suggested in the labelling of the dietary supplement.

We hereby confirm that this letter and the enclosed notification contain trade secret or otherwise confidential information that should not be disclosed to the public pursuant to 21 C.F.R. § 20.61 and 190.6(e). More specifically, the Notification contains valuable data and information that DuPont has held in strict confidence and has not disclosed to any member of the public. A letter identifying such confidential and propriety information and their location(s) within the document will be provided to the Office of Nutritional Products, Labeling, and Dietary Supplements of the Center for Food Safety and Applied Nutrition at a later date.

Respectfully,

A handwritten signature in cursive script, reading "M. Liebergesel".

Dr. Matthias Liebergesel
Global Regulatory Affairs Manager
DuPont Applied BioSciences

SECTION 1

The name and complete address of the manufacturer or distributor of the dietary supplement that contains a new dietary ingredient, or of the new dietary ingredient.

The manufacturer of the new dietary ingredient is:

E.I. du Pont de Nemours and Company

Direct correspondence to:

Dr. Matthias Liebergesell
Regulatory Affairs Manager
DuPont Applied BioSciences
Experimental Station 356/244B
Rt. 141 & Powder Mill Road
Wilmington, DE 19803-0356
Tel.: 302-695-7103
Fax: 302-695-7056
Matthias.Liebergesell@usa.dupont.com

SECTION 2

The name of the new dietary ingredient.

EPA-rich triglyceride oil. EPA refers to Eicosapentaenoic acid.

SECTION 3

Description of the dietary supplement or dietary supplements that contain the dietary ingredient including (i) the level of the dietary ingredient in the dietary supplement, and (ii) the conditions of use recommended or suggested in the labeling of the dietary supplement, or if no conditions of use are recommended or suggested in the labeling of the dietary supplement, the ordinary conditions of use of the supplement.

DuPont's EPA-rich triglyceride oil is intended for use in dietary supplements to provide omega-3 polyunsaturated fatty acids, and specifically, eicosapentaenoic acid (EPA). It contains natural tocopherols and other safe and suitable components for stabilization and will be sold in forms suitable for dietary supplements. Consumption of up to 2 grams (g) of EPA per day would be recommended or suggested in the labeling of the dietary supplement. Thus, use of DuPont's EPA-rich triglyceride oil in accordance with label recommendations would result in a maximum EPA intake of 2 g/day.

SECTION 4

The history of use or other evidence of safety establishing that the dietary ingredient, when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe, including any citation to published articles or other evidence that is the basis on which the distributor or manufacturer has concluded that the dietary supplement will reasonably be expected to be safe.

DuPont intends to produce a triglyceride oil that has a high content of omega-3 fatty acids, specifically, more than 35% of the triglyceride oil is EPA. The oil is obtained through a fermentation process using *Yarrowia lipolytica*, a yeast with a history of safe use as a food production microorganism. Information related to the production of the oil is provided in Sections 4.1 through 4.5.

4.1 *Yarrowia lipolytica* Characterization

4.1.1 Identification/taxonomic classification

- *Yarrowia lipolytica* ATCC #20362 (Wickerham *et al.*) van der Walt et von Arx, teleomorph; deposited by Bioteknika Int'l. as *Candida lipolytica* (Harrison) Diddens et Lodder, anamorph
- Biosafety Level: 1

4.1.2 Occurrence in Nature and History of Commercial Use

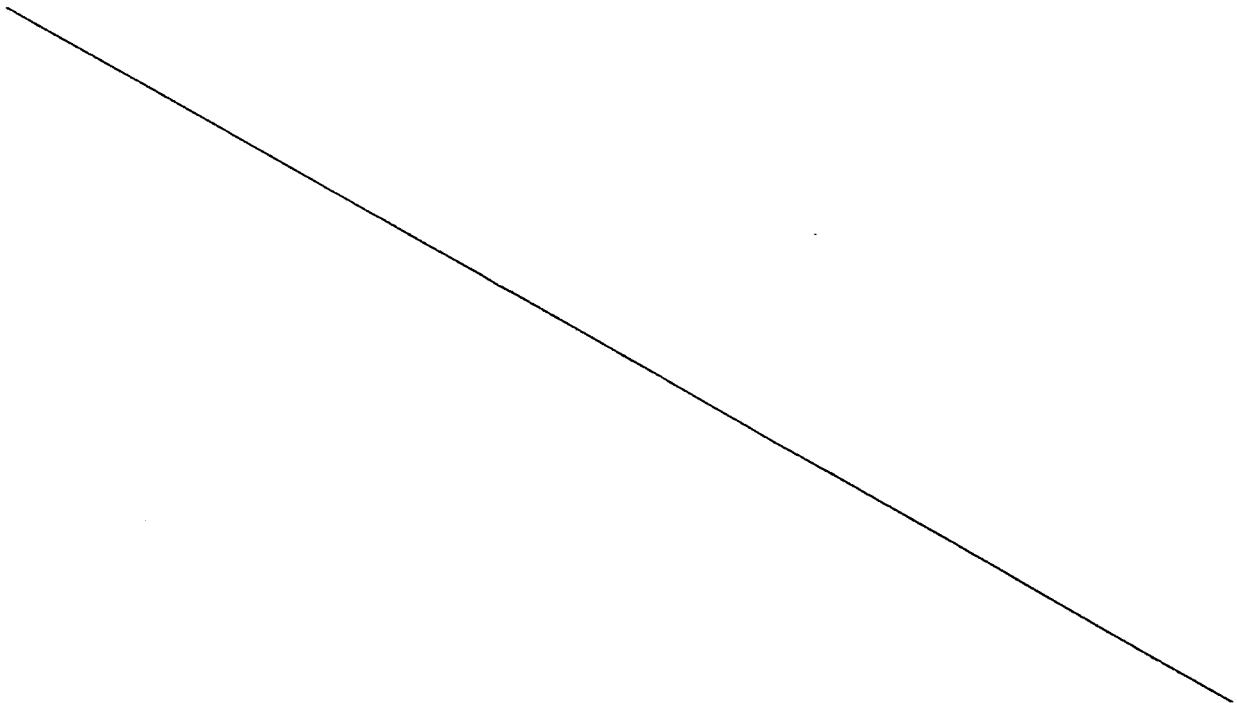
Yarrowia lipolytica is a hemiascomycetous yeast that is readily isolated from various food products. It was originally classified as a *Candida lipolytica* until the perfect form (sexual stage) was identified, and then it was reclassified first as *Endomycopsis lipolytica*, then as *Saccharomycopsis lipolytica* and finally as *Y. lipolytica*. It is not considered to be pathogenic. Intravenous injection into mice was not associated with any mortality and no viable cells could be recovered after 6 days. It is a strict aerobe, unable to survive under anaerobic conditions with a maximum growth temperature of ~32°C (DuPont, 2006).

Y. lipolytica is permitted as secondary direct food additive for fermentation production of food grade citric acid (21 CFR 173.165). It has also been produced commercially as a source of single cell protein for animal feeds (Toprina) and to produce γ -decalactone as a flavoring agent. Other applications include production of wax esters, 2-ketoglutaric, 2-hydroxyglutaric and

isopropylmalic acids and secretion of heterologous proteins, including several food enzymes. *Y. lipolytica* secretes lipases and proteases that contribute to the ripening of dairy products. Its lipase may also be useful for the synthesis of 2,4-dimethylglutaric acid monoesters and transesterification of meso-cyclopentane diols (DuPont, 2006).

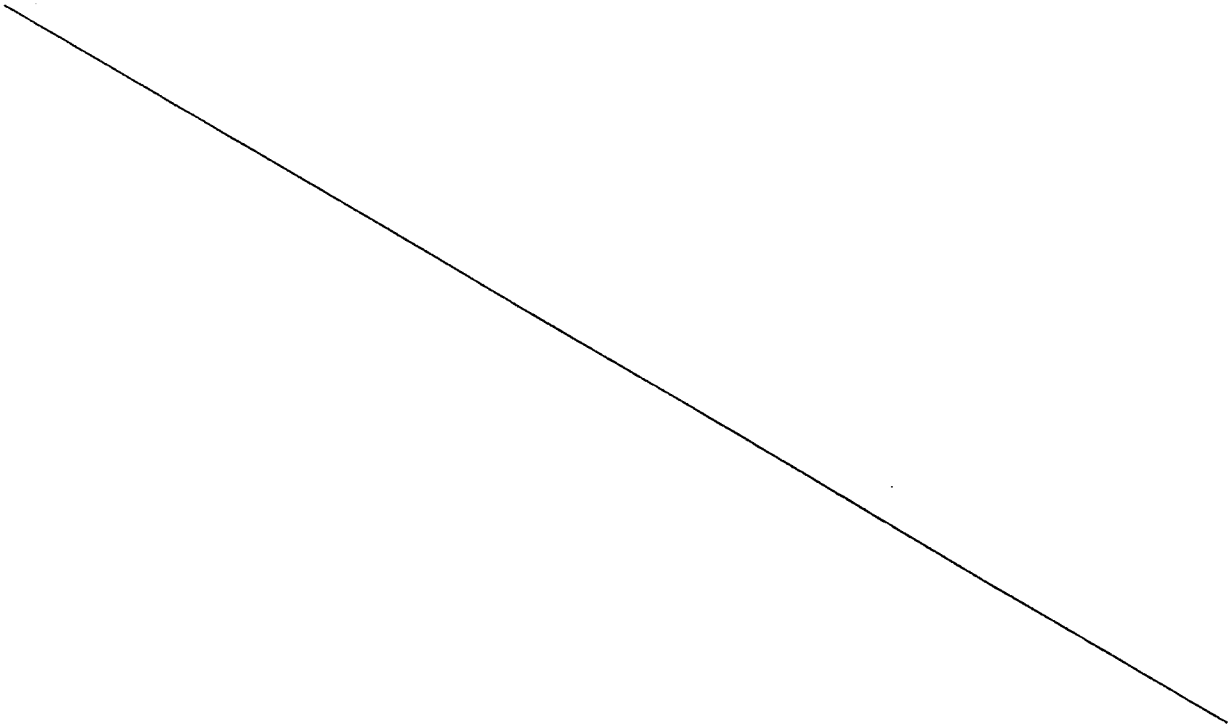
Oleaginous strains can accumulate more than 30% dry cell weight (dcw) as oil when starved for nitrogen in the presence of excess glucose or alkanes. The composition of the oil depends on the carbon source. Oil from glucose-grown cells is comprised mostly of triglycerides and a small amount of sterols. Oleic acid is the prominent fatty acid. Oil from oleic acid-grown cells contains more sterols and oleic acid than glucose-grown cells (DuPont, 2006).

The *Y. lipolytica* genome has been sequenced. It is comprised of 21Mb DNA containing 6650 open reading frames (ORFs) arranged on 6 chromosomes (DuPont, 2006).



Pages 6 through 14

**REDACTED IN ITS ENTIRETY
CONTAINS
TRADE SECRET
CONFIDENTIAL
COMMERCIAL
INFORMATION**



5.0 SAFETY OF THE MICROORGANISM UTILIZED IN THE PRODUCTION OF EPA-RICH TRIGLYCERIDE OIL

Yarrowia lipolytica is a dimorphic hemiascomycetous yeast that is readily isolated from various food products. It was originally classified as a *Candida lipolytica* until the perfect form (sexual stage) was identified, and then it was reclassified first as *Endomycopsis lipolytica*, then as *Saccharomycopsis lipolytica* and finally as *Y. lipolytica*. All strains are heterothallic (mating types MATA and MATB) and both haploid and diploid cells are vegetatively stable. Mating frequencies are very low and dependent on genetic background, growth medium, cell density, growth phase and temperature (DuPont, 2006). *Y. lipolytica* is not considered pathogenic (Holzschu *et al.*, 1979). Intravenous injection into mice was not associated with any mortality and no viable cells could be recovered after 6 days (DuPont, 2006).

Y. lipolytica is a strict aerobe with a maximum growth temperature of approximately 32 °C, and is able to utilize aliphatic carbon sources, including fatty acids and alkanes (DuPont, 2006;

Kerscher *et al.*, 2002; Madzak *et al.*, 2004). It has been used for numerous biotechnological applications. Traditional roles include processing of cheese and other dairy products, but it has also been exploited for the production of single cell protein for animal feeds, for the production of organic compounds like citric acid and α -ketoglutaric acid, and for the bioconversions of aliphatic compounds (Arzumanov *et al.*, 2000; Chernyavskaya *et al.*, 2000; Suzzi *et al.*, 2001; Vannini *et al.*, 2001).

Y. lipolytica differs considerably from other ascomycetous yeasts on the genetic level (DuPont, 2006). Its secretory apparatus (importance of the co-translational pathway, high efficiency, and low overglycosylation) make it closer to mammalian cells than to many other yeasts in some regards, and thus make it a useful host, especially for the production of complex proteins. Vectors and recipient strains are available and easy to handle, and amplification systems and the potential for scaled up production has been studied (Madzak *et al.*, 2004).

Oleaginous strains can accumulate more than 30% dcw as oil, when starved for nitrogen in the presence of excess glucose or alkanes. The composition of the oil depends on the carbon source. Oil from glucose-grown cells is comprised mostly of triglycerides and a small amount of sterols. Oleic acid is the prominent fatty acid. Oils from oleic acid-grown cells contain more sterols and oleic acid than glucose-grown cells (DuPont, 2006).

Y. lipolytica is permitted as secondary direct food additive for fermentation production of food grade citric acid (21 CFR 173.165). It has also been produced commercially as a source of single cell protein for animal feeds (Toprina) and to produce γ -decalactone as a flavoring agent. Other applications include production of wax esters, 2-ketoglutaric, 2-hydroxyglutaric and isopropylmalic acids and secretion of heterologous proteins, including several food enzymes. *Y. lipolytica* secretes lipases and proteases that contribute to the ripening of dairy products. Its lipase may be also useful for the synthesis of 2,4-dimethylglutaric acid monoesters and transesterification of meso-cyclopentane diols (DuPont, 2006).

The *Y. lipolytica* genome has been sequenced. It is comprised of 21Mb DNA containing 6650 ORFs arranged on 6 chromosomes. It is considered to be amenable for genetic development and to be a reliable and versatile host for heterologous production as it combines performance and regularity (Ogrydziak, 1988; Muller *et al.*, 1998; Kerscher *et al.*, 2002; Madzak *et al.*, 2004). As of 2004, forty-two heterologous proteins have been produced using *Y. lipolytica* (Madzak *et al.*, 2004).

6.0 OVERVIEW OF LONG CHAIN POLYUNSATURATED FATTY ACIDS

6.1 Overview

Long chain polyunsaturated fatty acids (LCPUFAs) are fatty acids of 20 carbons or more in length with two or more methylene-interrupted double bonds in the *cis* position. PUFAs can be grouped into two main families, omega-6 (or n-6) or omega-3 (or n-3), depending on the position of the first double bond proximate to the methyl end of the fatty acid. PUFAs are important structural components, modulating membrane fluidity and permeability and serving as precursors for a number of biologically active molecules such as eicosanoids, growth regulators, and hormones (Uauy *et al.*, 1996; Jump, 2002; La Guardia *et al.*, 2006).

Humans lack the enzymes required for the synthesis of the essential fatty acids linoleic acid and α -linolenic acid (Gebauer *et al.*, 2006). However, questions exist whether α -linolenic acid is essential *per se*, or whether eicosapentaenoic acid (EPA), which is derived from α -linolenic acid, and docosahexaenoic acid (DHA), which is in turn produced from EPA, are necessary (Spector, 1999). Thus, linoleic acid and α -linolenic acid must be supplied in the diet (Truska *et al.*, 2006). Although EPA and DHA can be synthesized from ALA, the extent of this metabolic conversion is limited at best. As such, dietary sources rather than metabolic conversion, constitutes the primary source of these fatty acids.

Common omega-6 fatty acids in the human diet are arachidonic acid, present in animal meat, and linoleic acid, present in vegetable oils, seeds, and nuts. Linoleic acid can be converted into arachidonic acid by desaturase enzymes. Major dietary sources of omega-3s include nuts, seeds, and vegetable oils containing α -linolenic acid (ALA), which can be converted to EPA and then DHA. In addition, EPA and DHA are consumed directly, with fatty fish serving as the most significant source. The α -linolenic acid, EPA, and DHA content of select foods is shown in Table 6-1.

Table 6-1 Omega-3 Fatty Acid Content of Select Foods (from DeFilippis and Sperling, 2006).

Food Item	EPA	DHA	ALA
Fish			
Catfish	Trace	0.2	0.1
Cod	Trace	0.1	Trace
Mackerel	0.9	1.4	0.2
Salmon			
Farmed	0.6	1.3	Trace
Wild	0.3	1.1	0.3
Canned	0.9	0.8	Trace
Salmon, Chinook	1.0	0.9	Trace
Swordfish	0.1	0.5	0.2
Tuna, Bluefin	0.3	0.9	-
Tuna, Light			
Canned in oil	Trace	0.1	Trace
Canned in water	Trace	0.2	Trace
Tuna, White			
Canned in oil	Trace	0.2	0.2
Canned in water	0.2	0.6	Trace
Shellfish			
Lobster	-	-	-
Mussels	0.2	0.3	Trace
Shrimp	0.3	0.2	Trace
Nuts and seeds			
Butternuts	-	-	8.7
Flaxseed	-	-	18.1
Walnuts	-	-	9.1
Plant oils			
Canola	-	-	9.3
Flaxseed	-	-	53.3

Measurements are expressed in grams per 100 grams (3.5 oz) of food item.

Trace – 0.1; (-) = 0 or no data

Adapted from the US Department of Health and Human Services

Estimates of dietary intake of omega-3 and omega-6 fatty acids vary within and between different populations, although generally, current intake of PUFAs consists primarily of n-6 fatty acids. The competition for desaturases and elongases in omega-3 and omega-6 PUFA metabolisms results in inverse effects on tissue concentrations of these fatty acids (Cunnane, 2003). The regulation of omega-6 and omega-3 fatty acid metabolism is complex, as EPA, DHA, arachidonic acid, α -linolenic acid, and even linoleic acid itself can inhibit the conversion of linoleic acid to arachidonic acid (Chen and Nilsson, 1993; Emken *et al.*, 1994; 1998; 1999; Sauerwald *et al.*, 1996).

It has been suggested that overall health may be improved by increasing omega-3 polyunsaturated fatty acid (PUFA) intake (Simopoulos, 1991). Estimates of paleolithic human diets suggest that the amounts of omega-3 PUFA and omega-6 PUFA were equal during early human evolution. Changes in food production have greatly altered those proportions in the last 100 to 150 years (Simopoulos, 1991). Intakes of omega-6 PUFAs are now up to 20 to 25 times higher than omega-3 PUFAs in the U.S. (Simopoulos, 1991; DeFilippis and Sperling, 2006). However, based on the limited studies in animals, children, and adults, a reasonable linoleic: α -

linolenic acid ratio of 5:1 to 10:1 has been recommended for adults (FAO/WHO, 1994). Numerous reviews in the literature have reported health benefits associated with even lower ratios.

The mean PUFA intake for men is 20.0 g/day and 16 g/day for women, with LA contributing 17.9 g/day and 13.5 g/day for total PUFA intake in men and women, respectively. The mean EPA + DPA + DHA intake was reported to be 0.17 g/day for men and 0.11 g/day for women. Grain products, meat, poultry, and fish account for most ALA intake (Ervin *et al.*, 2004; Food Surveys Research Group U, 2005). NHANES III reported a median intake of EPA + DHA of 0 and < 1 g/day of ALA. Meat, poultry, and fish contribute all of the EPA and DHA in women's diet and most of the intake in men, where eggs also contribute (Food Surveys Research Group U, 2005).

In the U.S., the Institute of Medicine (IOM), a subdivision of the National Academies of Sciences (NAS), made intake recommendations in its report on macronutrients (IOM, 2005) for omega-3 fatty acids and long-chain omega-3 fatty acids, DHA and eicosapentaenoic acid (EPA). The IOM Report sets an adequate intake (AI) for α -linolenic acid (ALA) of 1.6 g per day for adult men and 1.1 g per day for adult women. The IOM Report further elaborates on the AI for ALA and concludes that, "EPA and DHA can contribute approximately 10 percent of the total omega-3 fatty acid intake and therefore up to this percent can contribute towards the AI for α -linolenic acid". In further recognition of the importance of DHA, the IOM Report concludes that the acceptable macronutrient distribution range (AMDR) for ALA "is set at 0.6 to 1.2 percent of energy" and "up to 10 percent of this range can be consumed as eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA)".

In addition, the IOM panel found that: "[a] growing body of literature suggests that higher intakes of α -linolenic, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) may afford some degree of protection against coronary heart disease (CHD). Because the physiological potency of EPA and DHA is much greater than that for α -linolenic acid, it is not possible to estimate one AMDR for all omega-3 fatty acids. Up to 10 percent of the AMDR can be consumed as EPA and/or DHA".

Gebauer *et al.* (2006) reported that the evidence base supports a dietary recommendation of approximately 500 mg/day of EPA and DHA for cardiovascular disease risk reduction. For treatment of existing cardiovascular disease, 1 g/day is recommended (Gebauer *et al.*, 2006).

Canada was the first country to make separate recommendations for omega-3 and omega-6 fatty acid consumption. Health and Welfare Canada recommended daily intakes of omega-3 PUFA in the 1.0 to 1.6 g range for adults older than 19 years, with an additional 0.05 to 0.16 g for pregnancy, and 0.25 g for lactation. In addition to the recommendations of the IOM and Health and Welfare Canada, many other organizations have now made recommendations for total long-chain omega-3 fatty acid intake. These values are summarized in Table 6-2.

Table 6-2 International Recommendations for long-chain omega-3 fatty acids (From Gebauer *et al.*, 2006).

Source	Date	Total n-3 % of energy	ALA g/day	EPA + DHA mg/day	n-6:n-3
NATO workshop ¹	1989	-	3	800	4:1
UK Committee on Medical Aspects of Food Policy ²	1994	0.2	-	100-200	-
ISSFAL workshop ³	1999	1	2.2	650	-
ANC (France) ⁴	2001	0.8-1	1.8	450 (DHA: 110-120)	5:1
Eurodiet ⁵	2000	-	2	200	-
Health Council of the Netherlands ⁶	2001	1	-	200	7.5:1
American Heart Association ⁷	2002	-	-	~ 1g/d (2 ° prev CHD)	-
US National Academies of Science, Institute of Medicine ⁸	2002	-	1.4	~ 140	-
European Society of Cardiology ⁹	2003	-	-	~ 1g/d (2 ° prev CHD)	-
WHO FAO ¹⁰	2003	1-2	-	400-1000 (12 fish meals/week)	-
ISSFAL ¹¹	2004	-	1.6	≥ 500	-
UK Scientific Advisory Committee on Nutrition ¹²	2004	-	-	Minimum 2 portions fish/week (1 oily); assumes 450 mg	-

Abbreviations: ALA, α -linolenic acid; ANC, Apports Nutritionnelles Conseilles; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAO, Food and Agriculture Organization; ISSFAL, International Society for the Study of Fatty Acids and Lipids; NATO, North Atlantic Treaty Organization; WHO, World Health Organization

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6.1.1 Eicosapentaenoic acid (EPA)

Eicosapentaenoic acid (EPA) is the primary omega-3 fatty acid substrate for *in vivo* synthesis of 3-series prostanoids and especially 5-series leukotrienes. EPA may be derived from direct dietary intake, from desaturation and elongation of α -linolenic acid or from oxidative shortening of DHA.

6.1.2 Docosahexaenoic Acid (DHA)

Docosahexaenoic acid (DHA) is associated with membrane structural phospholipids, particularly those of the cerebral cortex, retina, testis and sperm. Omega-3 fatty acids, and DHA in particular, can increase cell membrane fluidity and deformability such as in erythrocytes (Simopoulos, 1991; Engler *et al.*, 1990). Its role as a nutrient essential to the optimum growth and development of visual and cognitive functions in premature and term infants is now recognized (Salem *et al.*, 1986).

6.2 Absorption, Distribution, Metabolism, and Excretion of Omega-3 Fatty Acids

The absorption, distribution, metabolism, and excretion of omega-3 fatty acids was recently reviewed by the Institute of Medicine. An overview is presented below.

6.2.1 Absorption

The digestion and absorption of omega-3 fatty acids is similar to that of other long-chain fatty acids (IOM, 2005). Following ingestion, triglycerides are generally emulsified in the stomach and duodenum, where they are mixed with bile salts and enzymatically hydrolyzed by pancreatic lipase. Pancreatic lipase has a high degree of specificity for the *sn*-1 and *sn*-3 positions of the triglyceride. The digested triglycerides are generally absorbed by the intestinal mucosa either as free fatty acids or as the *sn*-2 monoglyceride.

EPA and DHA are incorporated into circulating triglycerides in the same proportion that they appear in the dietary oil (Bronsgest-Schoulte *et al.*, 1981). Their *in vivo* peroxidation is the same as that seen for oleate or linoleate (Higdon *et al.*, 2000). EPA and DHA show specificity for different phospholipids (Mori *et al.*, 1987). Cholesterol esters become enriched with EPA but not with DHA (Holub *et al.*, 1987).

6.2.2 Distribution

The intestinal mucosa cells absorb the free fatty acids and *sn*-2 monoglycerides and convert these components into new triglycerides and phospholipids. Triglycerides accumulate in large

1 mm droplets containing about 88% triglyceride, 8% phospholipids, 3% cholesterol ester, and 1% to 2% protein. These droplets are ejected from gut mucosal cells by exocytosis as chylomicrons. Chylomicrons leave the intestine with lymph and enter the bloodstream *via* the thoracic duct.

6.2.3 Metabolism

Fatty acids are building blocks for new membranes and provide an energy-rich fuel source for cellular metabolism. In adipose cells, for example, free fatty acids produced by lipoprotein lipase are converted to triglycerides and stored for later use. When fat is needed for fuel, adipose tissue triacylglycerol undergoes lipolysis and free fatty acids are released into the circulation bound to albumin. Fatty acids then undergo β -oxidation to produce 2-carbon substrates from the Krebs's cycle. The Krebs's metabolic cycle produces CO_2 and NADPH. NADPH is then oxidized in mitochondria to produce ATP, releasing two electrons added to O_2 to make H_2O . The ATP produced in this way provides energy for all cellular functions (IOM, 2005).

The biosynthesis of long-chain polyunsaturated fatty acids from essential 18-carbon fatty acid precursors α -linolenic acid and linoleic acid obtained from the diet involves the sequential addition of 2-carbon units and desaturations catalyzed by $\Delta 5$ - and $\Delta 6$ -desaturases. The ability to convert α -linolenic acid to EPA and DHA and the pathways utilized in this process differ among mammalian species (IOM, 2005). This process is discussed in greater detail in Section 5.3.

6.2.4 Excretion

Fatty acids are almost completely absorbed and either oxidized to carbon dioxide and water, incorporated into tissue lipids, or utilized in eicosanoid synthesis. Small amounts of omega-3 fatty acids are lost during sloughing of skin and other epithelial cells.

6.3 Biosynthesis of Omega-3 Fatty Acids

6.3.1 Overview

The liver is the primary organ of lipid metabolism in humans. The first step in the biosynthesis of 20-carbon fatty acids is desaturation of the essential fatty acids linoleic or α -linolenic acid at the $\Delta 6$ position, followed by elongation. These 20-carbon products are desaturated by a $\Delta 5$ desaturase to produce ARA or EPA, respectively. The $\Delta 6$ -desaturation step is rate limiting in this metabolic pathway (Bernert and Sprecher, 1975; Yamazaki *et al.*, 1992; Willis and Browse, 1999).

Conversion of dietary ALA into EPA is limited, and efficacy of LCPUFA synthesis decreases down the cascade of ALA conversion such that DHA synthesis from ALA is even more restricted than that of EPA (Brenna, 2002; Sinclair *et al.*, 2002). Normally, most dietary LA and ALA are β -oxidized to provide energy and only a small portion of them (3.0% and 1.5%, respectively) are converted to longer PUFAs to meet the metabolic needs (Cunnane and Anderson, 1997). PUFA formation can be further hampered by other dietary components, hormonal imbalances, or certain chronic diseases (Huang *et al.* 2004). However, the synthesis of EPA and DHA from α -linolenic acid is not efficient, particularly when diets are rich in linoleic acid, which competes with α -linolenic acid for elongation and desaturation enzymes. As a result, it is usually necessary to obtain these acids directly from the diet in order to attain nutritionally adequate levels (Green, 2004).

6.3.2 The $\Delta 6$ -Desaturase/Elongation Pathway

Biosynthesis of LCPUFAs from their C18-PUFA precursors occurs *via* the conventional aerobic or $\Delta 6$ -pathway in most LCPUFA-synthesizing eukaryotes. LCPUFA biosynthesis starts with linoleic acid, which is either desaturated at the $\Delta 6$ -position and progresses down the omega-6 pathway, or is desaturated at the $\Delta 15$ -position to produce α -linolenic acid, and progresses down the omega-3 pathway. Subsequent desaturation and elongation steps occur in each pathway, carried out by shared enzymes. Fatty acids are linked either to phosphatidylcholine or coenzyme A. All desaturations occur to fatty acids coupled to phosphatidylcholine while elongations occur to fatty acids coupled to CoA. Enzymes termed acyltransferases are responsible for shuttling fatty acids between the phosphatidylcholine and CoA pools (Robert, 2005).

6.3.2.1 EPA synthesis

The first three steps in the production of EPA involve a $\Delta 6$ -desaturation of α -linolenic acid to SDA, a chain elongation to form omega-3-eicosatrienoic acid (ETA), and a $\Delta 5$ -desaturation to form EPA. Alternate pathways have been identified in other organisms (*e.g.*, some protists, including *Euglena gracilis*) and in some mammalian tissues, namely testis of rats and humans. In this pathway, the essential 18-carbon fatty acids are elongated to 20-carbon chain lengths followed by consecutive $\Delta 8$ - and $\Delta 5$ -desaturations (Huang *et al.*, 2004; Green, 2004; Hulanicka *et al.*, 1964; Albert and Coniglio, 1977; Albert *et al.*, 1979).

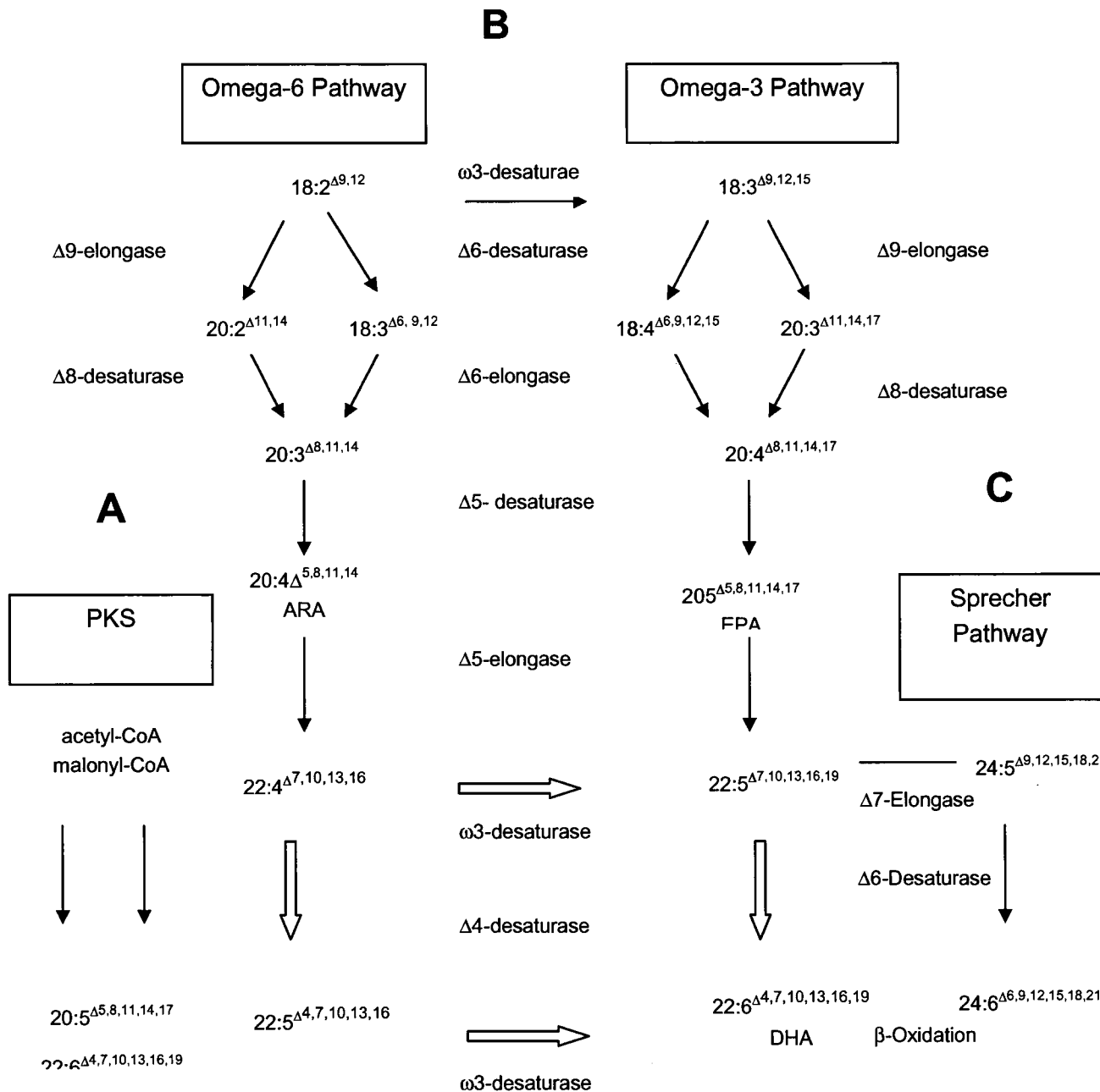
6.3.3 Alternate Pathways

Plants convert oleic acid (18:1) to linoleic acid (18:2) and linolenic acid [18:3(n-3)], however mammals lack the $\Delta 12$ - and omega-3 desaturase activities and obtain linoleic acid and α -linolenic acid from their diets (Bergé and Barnathan, 2005). Other organisms use a pathway mediated by the activities of $\Delta 9$ -elongase and $\Delta 8$ -desaturase. This $\Delta 8$ -desaturase activity has

also been observed in breast cancer and glioma cell lines (Grammatikos *et al.*, 1994; Bardon *et al.*, 1996; Cook *et al.*, 1991). Some marine bacteria and thraustochytrids are able to synthesize eicosapentaenoic acid and docosahexaenoic acid anaerobically by a completely different polyketide-like pathway (Metz *et al.*, 2001) that does not involve desaturation/elongation. Others utilize a group of enzymes designated the omega-3-desaturases that can catalyze the conversion of omega-6 to omega-3 (e.g., linoleic acid to α -linolenic acid, arachidonic acid to EPA). This pathway occurs frequently in lower organisms including algae, fungi, mosses, and worms, however, mammals lack both the omega-6- and the omega-3-desaturases (Meyer *et al.*, 2003).

An overview of the various pathways leading to EPA and DHA in microorganisms, plants, and animals is shown in Figure 6-1.

Figure 6-1 Alternative routes leading to EPA and DHA in microorganisms, plants, and animals (adapted from Meyer *et al.*, 2003)



(A) Polyketide synthase (PKS) systems producing either EPA or DHA (in marine bacteria (and possibly *Schizochytrium*)). (B) The omega-3- and omega-6 pathways of LCPUFA biosynthesis, including the routes that use $\Delta 9$ -elongase and $\Delta 8$ -desaturase. (C) The Sprecher Pathway operating in mammals. The omega-3-desaturase(s) may accept further omega-6 substrates in addition to the arrows included in the figure. It should be noted that switching from the omega-6 to the omega-3 track is not a universal alternative. It occurs frequently in lower organisms (algae, fungi, mosses, worms), but not in mammals, which lack both the omega-6 and the omega-3-desaturases.

6.3.4 Microbial Production of Polyunsaturated Fatty Acids

As noted previously, fatty fish serve as the major source of dietary EPA and DHA in the human diet. However, certain populations do not consume fish because of concerns about environmental contaminants (e.g., mercury and polychlorinated biphenyls), taste preferences, or ethical reasons (Gebauer *et al.*, 2006). In addition, there is a demand of PUFA sources that are more economical and sustainable than fish oils (Huang *et al.*, 2004). Recent progress in the cloning and identification of genes encoding the PUFA biosynthetic enzymes from different organisms has provided a potential means to produce long-chain PUFA-rich transgenic oils (Huang *et al.*, 2004). Food products enriched with microorganism derived oils offer an alternate source without these concerns, making it easier for individuals to meet the recommendations for omega-3 fatty acids.

Microalgae are primary producers of omega-3 fatty acids, however, certain characteristics of these organisms (e.g., photoautotrophic growth) make the commercial use of these organisms unfeasible (Wen and Chen, 2003). However, researchers have identified the genes involved in the biosynthesis of LCPUFAs from these organisms and have coexpressed these genes in other host systems, including plants and yeast that are more amenable to fermentation, efficient large-scale production, and less expensive isolation and refinement of oils, thus providing alternative sources of LCPUFAs (Truska *et al.*, 2006).

Sequences encoding virtually all of the enzyme activities involved in microbial PUFA synthesis have been isolated, identified, and expressed in a variety of heterologous hosts (Napier *et al.*, 1999). The specificity of these microbial enzymes allows them to cooperate in a processive manner, while the use of enzymes restricted to a single step in the biosynthetic pathway provides a means to reduce the formation of unwanted byproducts with unknown physiological effects (Meyer *et al.*, 2004).

As described in Section 3.0, DuPont intends to produce an EPA-rich triglyceride oil through heterologous expression of enzymes involved in the various pathways for LCPUFA synthesis in microorganisms in a bioengineered strain of *Y. lipolytica*.

Numerous authors have described the isolation of microbial enzymes involved in the different LCPUFA-biosynthesis pathways, including those used by DuPont in the current application, and have successfully expressed these heterologous proteins in a variety of yeast and plant host systems. For example, Kinney *et al* (2004) used a set of six genes, including three (a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, and a $\Delta 6$ elongase) from *M. alpina* and a $\Delta 17$ -desaturase from *Saprolegnia diclina* in soybean somatic embryos leading to the accumulation of up to 3% DHA.

The production of gamma-linolenic acid was achieved by the concurrent expression of $\Delta 12$ - and $\Delta 6$ -desaturases from *Mortierella alpina* (Huang *et al.*, 1999). ARA and EPA production from exogenously supplied fatty acid was accomplished by the expression of $\Delta 6$ - elongase and a $\Delta 5$ -

desaturase from this same fungus (Huang *et al.*, 1999; Parker-Barnes *et al.*, 2000). Beaudoin *et al.* (2000) and Domergue *et al.* (2002) achieved the synthesis of small amounts of ARA and EPA through the expression of these genes in yeast, commencing with a $\Delta 6$ -desaturation step. Meyer *et al.* (2004) reconstituted DHA biosynthesis in a yeast using either desaturases from the diatom *Phaeodactylum tricornutum* and the protist *Euglena gracilis* together with an elongase from the fish *Oncorhynchus mykiss*, or an elongase from the diatom *Thalassiosira pseudonana* and an elongase from the algae *Ostreococcus tauri*. These gene sets converted exogenously supplied stearidonic acid to DHA, however, unwanted side products were also formed.

Previous attempts to assemble the $\Delta 6$ -pathway in yeast and plants met with limited success, likely due to the failure to transfer enough of the initially formed $\Delta 6$ -desaturated C18-PUFAs from their phosphatidylcholine substrate to the acyl-coenzyme A pool where the subsequent elongation occurs (Beaudoin *et al.*, 2000; Domergue *et al.*, 2003). However others have suggested that heterologous expression of genes originating from different and often distantly related organisms could result in a poor spatial coordination of the enzymes on the endoplasmic reticulum, which could in turn cause inefficient transfer of substrates between them (Truska *et al.*, 2006).

Qi *et al.* (2004) used the $\Delta 9$ elongase from *Isochrysis galbana*, the $\Delta 8$ -desaturase from the protist *Euglena gracilis*, and the $\Delta 5$ desaturase from *M. alpina* to produce fatty acids in a plant species (*Arabidopsis thaliana*) and suggested that the alternative $\Delta 8$ -desaturation pathway may be a more appropriate choice for genetic modification of plants rather than the conventional $\Delta 6$ -desaturase/elongation route. This expression of the alternative $\Delta 8$ -pathway represents an important advance in demonstrating that particular combinations of enzymes may differ in their effectiveness in producing LCPUFAs. The authors were able to engineer *A. thaliana* plants by coexpression of a gene encoding $\Delta 8$ -PUFA elongase from the microalgae *Isochrysis galbana*, a gene encoding $\Delta 5$ -desaturase from *Mortierella alpina* and a gene encoding $\Delta 8$ -desaturase from *E. gracilis*. It is likely that the two consecutive desaturation steps in this $\Delta 8$ -pathway contributed to the increased efficiency of LCPUFA synthesis, compared with the $\Delta 6$ -pathway, because these reactions can occur sequentially while the acyl group remains on the phosphatidylcholine substrate.

Fungal and algal $\Delta 6$ -desaturases function on phospholipid-linked (mostly phosphatidylcholine-linked) LA or ALA substrates, whereas the PUFA-specific elongase requires its substrates to be present in the acyl-CoA pool. Yeast and plant model systems demonstrate inefficient transfer of the $\Delta 6$ -desaturated products from the phospholipids to the acyl-CoA pool due to a lack of suitable acyl transferase in these organisms (Domergue *et al.*, 2003). Thus, enzymes that are involved in the transfer of phospholipid-linked PUFAs to the acyl-CoA pools are desirable.

Recently, Picataggio *et al.* (2005) and Damude *et al.* (2006) of DuPont described the use of the oleaginous yeast *Yarrowia lipolytica* as a host for the production of omega-3 fatty acids using strains expressing heterologous desaturases, elongases and acyltransferases.

Altogether, these experiments demonstrated that the reconstitution of the biosynthetic pathways in a heterologous host was feasible, the heterologous condensing enzymes were compatible with the other components of the synthetic complex.

6.3.5 Dietary Effects of Supplementary Omega-3 Fatty Acids

Arterburn *et al.* (2006) reviewed the tissue distribution, interconversion, and dose response data on omega-3 fatty acids. The available data suggests that supplementation of the typical Western diet with even large doses of ALA results in only marginal changes in ALA plasma concentrations, small increases in plasma EPA, and no effects on plasma DHA. These data are consistent with the high oxidation rate and low fractional conversion rates for the n-6 fatty acids known to occur in humans.

EPA concentrations in plasma increase in response to dietary EPA, but DHA levels remain unaffected. Dietary DHA results in a dose-dependent, saturable (at approximately 2 g/day) increase in plasma DHA concentrations and modest increases in EPA concentrations. Plasma DHA concentrations equilibrate in approximately 1 month and then remain at steady state throughout supplementation. Both dietary DHA and EPA reduce plasma arachidonic acid concentrations. Tissue concentrations of DHA and EPA can also be elevated through dietary supplementation. Only minute quantities of EPA are generally present in tissues. DHA concentrations range from 5 to 30-fold in most organs, and several hundred-fold more in brain and retina.

As reviewed in Arterburn *et al.* (2006), EPA is more rapidly cleared from plasma than DHA. Plasma EPA concentrations decreased rapidly after cessation of omega-3 supplementation, and approached baseline levels within 4 weeks, whereas DHA concentrations remained above baseline levels after 24 weeks. Both DHA and EPA decrease more rapidly in plasma cholesterol esters than in phospholipids. EPA washout from red blood cells is also faster than that of DHA. Whereas DHA accumulates predominantly in phospholipids, EPA is more equally distributed between neutral lipids (sterol esters and triacylglycerols) and phospholipids. This differential distribution may be linked to differences in the kinetics of washout and their saturation dynamics in plasma and availability to tissues.

Arterburn *et al.* (2006) suggested that the most predictable way to increase a specific long-chain omega-3 fatty acid in plasma, tissues, or human milk is to supplement with the fatty acid of interest.

6.4 Physiological Role of Omega-3 Fatty Acids

As noted in Section 6.1, polyunsaturated fatty acids are important structural components, modulating membrane fluidity and permeability. Docosahexaenoic acid (DHA) is associated with membrane structural phospholipids, particularly those of the cerebral cortex, retina, testis and sperm, and as such, inadequate intakes of omega-3 fatty acids are associated with impaired visual and neural function, as well as a number of other impaired health endpoints relating to the effects of omega-3 fatty acids on various physiological processes. There is no accepted cut-off concentration of plasma or tissue concentrations below which these functions are impaired (IOM, 2005).

In addition to structural roles, omega-6 and omega-3 fatty acids serve as precursors to two distinct classes of eicosanoid metabolites whose hormone-like properties play critical roles in modulating the response of several homeostatic and immune mechanisms. The omega-6 acid arachidonic acid (ARA) is the precursor of the 2-series of cyclooxygenase generated prostanoids (prostaglandins and thromboxanes) and the 4-series of lipoxygenase-generated leukotrienes, while the omega-3 fatty acid EPA is a precursor of the 3-series prostanoids and the 5-series leukotrienes. Corresponding members of the two series often have differing or even opposing activity; with the ARA series generally activating immune and inflammatory processes, the DHA/EPA series generally decreasing them (Simopoulos, 1991). The relative balance among members of these two eicosanoid families is important in determining their net local effect. As discussed in Section 5.3, the synthesis of omega-6 and omega-3 PUFAs is mediated by the same enzymes, which generally appear to have no particular preference for substrate. Thus, the balance of intake of omega-6 and omega-3 fatty acids will determine the types and amounts of eicosanoids in the body (Sayanova and Napier, 2004).

Eicosanoids derived from omega-6s are generally pro-inflammatory and pro-aggregatory, whereas those from omega-3s are predominately anti-inflammatory and inhibit platelet aggregation (Simopoulos, 1999). This may account for the cardioprotective effects of omega-3's (DeFilippis and Sperling, 2006). In addition to markers of vascular inflammation, omega-3s may beneficially influence other factors related to cardiovascular disease risk, including ventricular arrhythmia's, thrombosis, triglycerides, apolipoprotein B, high-density lipoprotein, adhesion molecule expression in plaque, platelet-derived growth factor, nitric oxide induced endothelial relaxation, and blood pressure (Kris-Etherton *et al.*, 2003; Simopoulos, 1999; Weber and Leaf, 1991; Weber, 1989; Harper and Jacobson, 2001).

Competition occurs between ARA and EPA (but probably not DHA) for prostaglandin and leukotriene synthesis at the cyclooxygenase and lipoxygenase level, EPA being a better substrate for lipoxygenase than for cyclooxygenase (Linscheer and Vergroesen, 1994). Ingested and derived DHA and EPA may partially replace ARA in cell membranes, especially those of platelets, erythrocytes, monocytes, neutrophils and liver cells (Simopoulos, 1991). In the case of DHA, competition with ARA would be expected to arise in two ways. Partial

replacement of ARA by DHA in membrane lipids may initially increase and then subsequently decrease the amount of ARA substrate available for prostanoid and leukotriene synthesis. Since the availability of dietary and derived ARA is comparatively high, competition at this level seems unlikely to assume significant proportions unless DHA intake is elevated substantially. The second avenue is *via* conversion of DHA to EPA [(Brossard, *et al.*, 1996) which in turn may directly compete with ARA for access to synthetic enzymes as stated earlier. Since EPA is a preferred substrate for these enzymes compared to ARA (Poisson *et al.*, 1992; Simopoulos, 1991; Linscheer and Vergroesen, 1994) competition at this level may be significant at relatively low EPA levels.

Accordingly, increased ingestion of omega-3 fatty acids may lead to:

1. decreased production of prostaglandin E₂ (PGE₂) metabolites;
2. decreased production of thromboxane A₂, which is a potent platelet aggregation and vasoconstriction agent;
3. decreased formation of leukotriene B₄, a potent leukocyte chemotaxis and adherence agent and inflammation factor;
4. increased formation of thromboxane A₃, which exhibits only weak platelet aggregation and vasoconstriction activity;
5. an increase in formation of prostacyclin E₃ (PGI₃) without a decrease in PGI₂ leading to an overall increase in prostacyclins which are vasodilators and inhibit platelet aggregation; and
6. increased formation of leukotriene B₅, a weak chemotactic and weak inflammatory agent.

6.4.1 Effects of Omega-3 Fatty Acid Supplementation on Cardiovascular Health

Epidemiological data and prospective randomized trials demonstrate omega-3 fatty acids offer protection against cardiovascular disease (DeFilippis and Sperling, 2006). It is believed these effects are mediated through production of eicosanoids, a class of bioactive molecules derived from omega-3s and omega-6s that include leukotrienes, prostaglandins, and thromboxanes. Thus, dietary intake of these fatty acids may also prove to be useful in moderating the risk of cardiovascular disease. In 2005, the Institute of Medicine (IOM) published *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids*, which contains an extensive review of the epidemiological and clinical evidence on the health benefits associated with omega-3 fatty acids (IOM, 2005). This report summarizes the data on the health benefits associated with diets containing omega-3 fatty acids and goes on to

state that a “growing body of literature suggests that diets higher in α -linolenic acid, EPA, and DHA may afford some degree of protection against CHD”.

Wang *et al.* (2006) recently reported the results of an evidence-based, systematic review of the literature on the effects of omega-3 fatty acids consumed as fish, or fish oils rich in EPA and DHA, or as alpha-linolenic acid, on cardiovascular disease outcomes and adverse effects associated with their consumption. Randomized controlled trials and prospective cohort studies that followed patients for at least 1 year and case control studies that reported intakes of omega-3 fatty acids or fish were included in the review. Evidence suggested that the increased consumption of omega-3 fatty acids from fish or fish oil supplements, but not from alpha-linolenic acid, reduces the rates of all-cause mortality, cardiac and sudden death, and possibly stroke, with stronger evidence of beneficial effects in secondary-prevention settings (patients with a history of cardiovascular disease) than in primary prevention settings (general population with no history of cardiovascular disease).

The most compelling evidence for the cardiovascular benefit provided by omega-3 fatty acids comes from 3 large controlled trials of 32,000 participants randomized to receive omega-3 fatty acid supplements containing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) or to act as controls. These trials showed reductions in cardiovascular events of 19% to 45%. For example, the Japan EPA Lipid Intervention Study (JELIS), 18,645 patients with hypercholesterolemia (70% women; mean age, 61 years) were randomly assigned to either statin alone or statin and pure EPA (1.8 g/d). During the 5-year study, EPA reduced major adverse CV events by 19%. Additionally, the JELIS trial established the safety and efficacy of combination therapy with EPA and a statin vs statin therapy alone for improving CV prognosis (Yokoyama *et al.*, 2007).

Balk *et al.* (2007) published the results of an evidence-based systematic review of the data related to the use of omega-3 fatty acids in a variety of clinical conditions. The authors reported that adequate evidence is available to attribute a health benefit to consumption of supplemental fish oil or dietary fish only with respect to a reduction in cardiovascular events, principally as secondary prevention. Furthermore, fish oils reduce serum triacylglycerol concentrations, reduce blood pressure by a small but significant amount, reduce corticosteroid use in patients with rheumatoid arthritis, and reduce the risk of non-Alzheimer dementia in certain persons and at various doses.

6.5 Safety of Omega-3 Fatty Acids

6.5.1 Overview

The safety of omega-3 fatty acids is supported by their long history of ingestion as a component of the human diet. Although the high LCPUFA doses used in animal studies ($>1\text{g/kg}$ body

weight/day) may produce adaptive non-specific effects in liver metabolism and histomorphology, these effects are related to the extra metabolic workload and are not relevant to exposure at much lower doses.

Adverse effects associated with omega-3 polyunsaturated fat consumption have been reported in a few special subpopulations (IOM, 2005). It has been suggested that individuals exhibiting glucose intolerance or diabetic conditions use caution with omega-3 fatty acids as some have required increased doses of hypoglycemic agents (Glauber *et al.*, 1988; Kasim *et al.*, 1988; Friday *et al.*, 1989; Stacpoole *et al.*, 1989; Zambon *et al.*, 1992). Clarke *et al.* (1990) reported increased episodes of nosebleeds in individuals with familial hypercholesterolemia during fish oil supplementation. High levels of eicosapentaenoic acid (EPA) content may reduce growth in infants (Carlson *et al.*, 1993).

The FDA critically evaluated available information on the safety on the omega-3 fatty acids, EPA and DHA, and identified three potential safety issues, namely prolongation of bleeding time, modification of glycemic control, and elevation of LDL cholesterol (US FDA, 1993). Similarly, the Societa Italiana di Nutrizione Umana (1996) and Hughes and Pinder (1997) reported that the intake of excessive omega-3 fatty acids may result in toxic effects, including increased bleeding rate, alteration of immune function, and formation of toxic lipoperoxides (Societa Italiana di Nutrizione Umana, 1996; Hughes and Pinder, 1997). It has been noted that the intake of omega-3 fatty acids should also include adequate quantity of vitamin E or tocopherols to limit oxidation of PUFAs by free radicals (Witting and Lee, 1975; Brude *et al.*, 1997; La Guardia *et al.*, 2006).

A more comprehensive discussion of the potential safety issues associated with omega-3 fatty acids is provided in Sections 6.5.2 through 6.5.5.

The results of numerous clinical studies published since that time indicate the DHA provided in fish or marine-derived oils at levels up to 6 g DHA/person/day would not be expected to produce adverse effects on these parameters (Kroes *et al.*, 2003). These results are consistent with the FDA conclusion in its 1997 menhaden oil decision that the combined consumption of up to 3 g of DHA + EPA/person/day poses no significant risk for bleeding time, produces no clinically significant effect on glycemic control, and is safe with respect to the effect on LDL cholesterol (FDA, 1997).

The US Department of Health and Human Services Agency for Healthcare Research and Quality (2004) identified 148 studies on omega-3 fatty acids that evaluated over 20,000 subjects for adverse effects. The most common side effects were gastrointestinal complaint, reported among 6.6% of patients taking omega-3s versus 4.3% in placebo groups. An increased incidence of bleeding was not observed, and only 1 of the 148 studies reported such an association in patients randomized to 6 g/day of omega-3. There were no reported deaths or life-threatening illnesses as a consequence of omega-3 consumption. No adverse effects were

reported in 77 of the 148 studies. Based on this review, the agency concluded that adverse effects related to consumption of fish oil or α -linolenic supplements appear to be minor.

Wang *et al.* (2006) recently reported the results of an evidence-based, systematic review of the literature on the effects of omega-3 fatty acids consumed as fish, or fish oils rich in EPA and DHA, or as alpha-linolenic acid, on cardiovascular disease outcomes and adverse effects associated with their consumption. Adverse effects were generally considered to be minor. Gastrointestinal side effects were the most often reported, but only 4% of complaints occurred at dosages of less than 3 g/day. GI symptoms increased to approximately 20% at a dose of 4 g/day. Nine studies included in the review addressed the risk of clinically significant bleeding episodes. Four of these studies reported no bleeding in either arm, while in the remaining five, no consistent association found between the omega-3 fatty acid dose and the risk of bleeding, even in those patients taking aspirin or warfarin.

Similarly, in their evidence-based systematic review of the data related to the use of omega-3 fatty acids, Balk *et al.* (2007) found that although only a limited number of the reviewed articles provided information on adverse events, and the reporting of adverse events in these studies was generally inadequate and incomplete (e.g., rates of adverse events were not systematically or consistently monitored and reported), no evidence of substantial adverse events related to omega-3 fatty acid consumption beyond mild gastrointestinal symptoms was found.

6.5.2 Effects on Bleeding Time

As reviewed in Section 6.4, a link between omega-3 polyunsaturated fatty acid intake and reduced risk of coronary heart disease has been suggested, with reduced platelet aggregation proposed as a possible mechanism. This effect may also result in prolonged bleeding time, although intervention studies examining the effects of omega-3 fatty acids on bleeding time have produced equivocal results (IOM, 2005).

Thorngren and Gustafson (1981) reported that ingestion of omega-3 fatty acids by individuals taking anticoagulants such as aspirin, warfarin, and coumadin may result in prolonged bleeding times. Collective analysis of numerous clinical studies with EPA/DHA supplements, ranging in dose levels from 2 to 15 g/day, has shown no dose response for EPA and DHA on bleeding times. None of the studies indicated excessive bleeding times, bleeding episodes, or bruising (IOM, 2005). Some studies have indicated an increased risk of hemorrhagic stroke among Greenland Eskimos with high EPA and DHA intakes (Kromann and Green, 1980; Kristensen, 1983; Pedersen *et al.*, 1999). While a prospective study in the U.S. showed no association between intake of ω -3 fatty acids and risk of hemorrhagic stroke, the median intakes ranged from only 0.077 to 0.481 g/day, which were considered to be relatively low levels of intake in the U.S. (Iso *et al.*, 2001; IOM, 2002). Increased lipid peroxidation and oxidative damage of erythrocytes, liver, and kidney membranes and bone marrow DNA have been reported in feeding studies with DHA in laboratory animals (Ando *et al.*, 1998; Yasuda *et al.*, 1999; Song

and Miyazawa, 2001; Umegaki *et al.*, 2001); however, consumption of vitamin E reportedly reduced or prevented the oxidative damage (Leibovitz *et al.*, 1990; Ando *et al.*, 1998; Yasuda *et al.*, 1999).

Bays (2007) reported that the clinical trials have shown high-dose fish oil omega-3 fatty acid consumption to be safe, even when concurrently administered with other agents that may increase bleeding, such as aspirin and warfarin. He noted that in certain clinical settings, it could be argued that the reported antiatherothrombotic effects of fish oils rich in omega-3 fatty acids are a benefit that outweighs the unproved bleeding risks, especially in select patients at high risk for thrombosis, which may include patients with acute atherosclerotic CAD.

Likewise, a recent comprehensive report by Harris (2007) concluded that virtually no increased risk for clinically remarkable bleeding has been seen in patients with doses of omega-3 fatty acids up to 7 g/d of DHA and EPA,.

6.5.3 Effects on LDL cholesterol

Prospective cohort studies and a trial of secondary prevention of ischemic heart disease suggested that intakes of greater than 1 g/day of omega-3 LCPUFAs offer protection from fatal ischemic heart disease (Hu *et al.*, 2002; Albert *et al.*, 1998, Anonymous, 1999). Likewise, a mixture of EPA and DHA has been shown to lower serum triacylglycerol concentrations, though a slight and, concurrent increase in LDL cholesterol concentrations may occur, particularly in hypertriacylglycerolemic subjects (Harris, 1989; 2003; Davidson *et al.*, 1997; Anonymous, 1999); Mori *et al.*, 2000; Montori *et al.*, 2000; Theobald *et al.* 2004) while favorable effects on lipoprotein particle distributions may occur (Li *et al.*, 2004). Increased LDL levels are associated with increased risk of cardiovascular disease, thus increases in LDL could counteract other beneficial cardiovascular effects associated with omega-3 oils.

Studies using purified EPA or EPA-rich oil generally have not reported increases in LDL cholesterol (Harris, 1997). DuPont's EPA-rich triglyceride oil would thus be anticipated to offer the beneficial health effects associated with omega-3 fatty acids without the potential LDL-increasing effects attributed to DHA.

A decrease in total VLDL particle concentrations has previously been reported after consumption of EPA and DHA-rich fish oil (Li *et al.*, 2004). In the 2005 study by Goyens and Mensink, however, the proportion of EPA in plasma phospholipids was significantly increased with the consumption of both experimental diets, whereas DHA was not affected. This suggested to authors that EPA may be responsible for the changes reported in VLDL concentrations after consumption of fish oil.

6.5.4 Effects on Glycemic Control

Montori *et al.* (2000) reviewed the data from eighteen trials including 823 type 2 diabetic subjects dosed with 3 to 18 g of fish oil per day for 12 weeks. Researchers found that fish oil supplementation in type 2 diabetics lowered triglycerides, raised LDL cholesterol, and had no statistically significant effect on fasting glucose or glycemic control.

6.5.5 Alteration of Immune Function

Suppression of various aspects of human immune function has been reported *in vitro* or *ex vivo* in peripheral blood mononuclear cells, in isolated neutrophils or monocytes, or in subjects provided omega-3 polyunsaturated fatty acids as a supplement or in experimental diets. The minimum dose observed for such an effect was 0.9 g/day EPA and 0.6 g/day DHA given as fish oil for 6 to 8 weeks to healthy adults (Cooper *et al.*, 1993). Single treatment studies suggest that intakes of EPA and DHA at levels 7 to 15 times the typical U.S. intake diminish the potential of the immune system to attack pathogens (Lee *et al.*, 1985; Schmidt *et al.*, 1989; Kelley *et al.*, 1998, 1999). These doses have also reportedly been associated with suppression of inflammatory responses. However, Blok *et al.* (1997) administered fish oil supplements (0, 3, 6, or 9 g/day fish oil supplement resulting in EPA intakes of 0, 0.81, 1.62, or 2.43 g/day and DHA intakes of 0, 0.16, 0.33, or 0.49 g/day) for 1 year to 58 healthy men. *Ex vivo* endotoxin stimulated production of interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , or IL-1Ra did not differ among treatment groups up to 6 months after supplementation was stopped, supporting a lack of long-term adverse effects of fish oil supplementation on cytokine activity.

Hawkes *et al.* (2002) examined the effects of supplementation with DHA-rich tuna oil on the human milk cytokines interleukin 1 β , interleukin 6, and tumour necrosis factor α . DHA levels in maternal plasma, human peripheral blood mononuclear cells (PBMCs), milk, and human milk cells (HMCs) were increased in a double-blind, prospective, randomized trial in which mothers of healthy full-term infants were asked to consume a nutritional supplement containing either 300 mg DHA + 70 mg EPA or 600 mg DHA + 140 mg EPA for 4 weeks. DHA concentrations reportedly increased in a linear manner. Despite the increased n-PUFA concentrations in relevant tissues, no perturbations in cytokine concentrations were observed. Side effects were reported by 7 to 15% of the subjects and included gas (n=4), fishy burps (n=4), unsettled babies (n=1), and rash (n=1).

The effects of dietary fatty acid supplementation from various sources on endothelial function and tone in healthy subjects were examined by Khan *et al.* (2003). Volunteers took oil supplements containing oleic acid rich sunflower oil, evening primrose oil, soybean oil, tuna fish oil (6% EPA acid and 27% DHA), or tuna/evening primrose oil mix for 8 months. Endothelium-dependent and endothelium-independent responses to iontophoresis of acetylcholine and sodium nitroprusside, respectively, were measured in the forearm skin using laser Doppler imaging. Acetylcholine responses were significantly improved after tuna oil supplementation

while significant positive correlations were also seen between acetylcholine responses and omega-3 fatty acid levels in the plasma and erythrocyte membrane phospholipids in subjects consuming the tuna oil supplements. No significant differences were observed with any of the other oils. Total cholesterol, red and white blood cell counts, hemoglobin, and hematocrit levels were not significantly affected by tuna oil supplementation. The authors concluded that modification of the diet involving a 6% increase in EPA acid and 27% DHA might have significant beneficial effects on cardiovascular function and health.

6.5.6 Dosing Recommendations:

Mozaffarian and Rimm (2006) reviewed the available scientific data on the cardiovascular and neurological health benefits of omega-3 fatty acid intake from fish or fish oil, as well as the possible adverse effects associated with environmental contaminants (i.e., mercury, dioxins, and polychlorinated biphenyls) associated with these commodities. Analysis of the data suggested that consumption of 1 to 2 servings of fish per week, particularly oily species higher in EPA and DHA, was associated with a reduced risk of coronary death (36% reduction, 95% confidence interval, 20%-50%; $p < .001$) and mortality (17% reduction, 95% confidence interval, 0%-32%; $p = .046$). Other clinical outcomes may also be favorably affected. For example, there was moderate evidence suggesting a reduced risk of ischemic stroke, and equivocal evidence that fish and fish oils produce modest benefits related to nonfatal myocardial infarctions, progression of atherosclerosis, postangioplasty restenosis, and recurrent ventricular tachyarrhythmias. Limited evidence suggests decreased risk of atrial fibrillation and congestive heart failure associated with intake of fish and fish oils. The data indicated that EPA and DHA intakes of 250 mg/day appear sufficient for primary prevention, with little additional risk reduction at higher intakes (Mozaffarian and Rimm, 2006). For individuals with coronary heart disease, 500 to 1000 mg/day of EPA and DHA is currently recommended to reduce CHD mortality (Kris-Etherton *et al*, 2002; Van de Werf, 2003; Mozaffarian and Rimm, 2006)

It is the position of the American Heart Association that “omega-3 fatty acids benefit the hearts of healthy people, and those at high risk of, or who have cardiovascular disease.” (American Heart Association, 2006)

The American Heart Association recommends patients without documented coronary heart disease consume a variety of fatty fish at least twice per week. Oils and foods rich in alpha-linolenic acid (flaxseed, canola and soybean oils; flaxseed and walnuts) should also be included, although the AHA notes that the extent of in vivo conversion to omega 3 fatty acids is small and that more studies are needed to show the beneficial effects of alpha-linolenic acid and heart disease. Patients with documented coronary heart disease are advised to consume about 1 g of EPA + DHA per day, preferably from fatty fish. Capsule form could be considered after consulting with a physician. Similarly, it is recommended that patients who need to lower triglycerides consume 2 to 4 g EPA + DHA per day provided as capsules under a physician's

care. However, as noted by Lee et al. (2008), the lower end of this dose range is ineffective in most patients, and thus clinicians should target between 3.0 and 4.0 g/d to achieve meaningful triglyceride lowering.

The AHA notes that patients taking more than 3 grams of omega-3 fatty acids from capsules should do so only under a physician's care since high intakes could cause excessive bleeding in some people. The AHA noted that increasing omega-3 fatty acid intake through foods is preferable, but that coronary artery disease patients may not be able to get enough omega-3 by diet alone. Likewise, people with high triglycerides, who need even larger doses, can also benefit from supplements. The availability of high-quality omega-3 fatty acid supplements, free of contaminants, is an important prerequisite to their use (American Heart Association, 2006).

A summary of these dosing recommendations is shown in Table 6-2.

Table 6-2 Summary of Omega-3 Dosing Recommendations

Recommending Organization/Researcher	Target Population	Amount Recommended	Suggested Benefit
Mozaffarian and Rimm, 2006	Patients without documented coronary heart disease (CHD)	250 mg EPA +DHA/day	Primary prevention of coronary heart disease(CHD)-related death
American Heart Association	Patients without documented coronary heart disease (CHD)	Eat a variety of (preferably fatty) fish at least twice a week. Include oils and foods rich in alpha-linolenic acid (flaxseed, canola and soybean oils; flaxseed and walnuts).	Omega-3 fatty acids reduce CVD risk through • decreased risk of arrhythmias, which can lead to sudden cardiac death • decreased triglyceride levels • decreased growth rate of atherosclerotic plaque • lower blood pressure (slightly)
Kris-Etherton <i>et al</i> , 2002; Van de Werf, 2003; Mozaffarian and Rimm, 2006	Individuals with coronary heart disease	500 to 1000 mg/day of EPA and DHA	Reduced CHD mortality
American Heart Association	Patients with documented CHD	Consume about 1 g of EPA+DHA per day, preferably from fatty fish. EPA+DHA in capsule form could be considered in consultation with the physician.	Omega-3 fatty acids reduce CVD risk through • decreased risk of arrhythmias, which can lead to sudden cardiac death • decreased triglyceride levels • decreased growth rate of atherosclerotic plaque • lower blood pressure (slightly)
American Heart Association	Patients who need to lower triglycerides	2 to 4 grams of EPA+DHA per day provided as capsules under a physician's care.	Omega-3 fatty acids reduce CVD risk through • decreased risk of arrhythmias, which can lead to sudden cardiac death • decreased triglyceride levels • decreased growth rate of atherosclerotic plaque • lower blood pressure (slightly)

7.0 RESULTS OF DUPONT SAFETY STUDIES

Nonclinical studies conducted by DuPont supporting the safety of EPA-rich triglyceride oil include a 28-day repeated dose toxicity study in rats and various genotoxicity studies (a bacterial reverse mutation assay, *in vitro* lymphoma assay, and *in vivo* mouse bone marrow micronucleus test). These studies are summarized in Sections 7.1 and 7.2.

7.1 Genotoxicity and Mutagenicity

7.1.1 Bacterial Reverse Mutation Assay

DuPont's EPA-rich triglyceride oil was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvrA* in the presence and absence of Aroclor-induced rat liver S9 (DuPont, 2009a). Dimethyl sulfoxide (DMSO) was selected as the solvent. The assay was performed in two phases, using the plate incorporation method. The first phase, the initial toxicity-mutation assay, was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. The second phase, the confirmatory mutagenicity assay, was used to evaluate and confirm the mutagenic potential of the test article. The positive control substance used for all strains with S9 activation was 2-aminoanthracene. In the absence of S9 activation, positive controls were 2-nitrofluorene (for strain TA98), sodium azide (for strains TA100 and TA1535), 9-aminoacridine (for strain TA1537), and methyl methanesulfonate (for WP2 *uvrA*).

Results are shown in Tables 7-1 and 7-2. No positive mutagenic response was observed in either the dose-range finding assay or the confirmatory mutagenicity assay at doses up to 5000 µg per plate. These results indicate that, under the conditions of this study, DuPont's EPA-rich triglyceride oil did not exhibit any mutagenic responses in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test article was concluded to be negative in this assay.

Table 7-1 Results of the Bacterial Mutagenicity Assay (Initial Toxicity Mutation Assay)

Test Article Id : H-28297
 Study Number : AC14XM.503.BTL
 Experiment No : B1

Average Revertants Per Plate $\pm$ Standard Deviation

Activation Condition	None									
Dose (μ g per plate)	TA98		TA100		TA1535		TA1537		WP2 uvrA	
Vehicle	16	$\pm$ 1	93	$\pm$ 1	10	$\pm$ 2	9	$\pm$ 1	22	$\pm$ 0
1.5	15	$\pm$ 1	103	$\pm$ 1	10	$\pm$ 4	12	$\pm$ 2	26	$\pm$ 10
5.0	16	$\pm$ 1	119	$\pm$ 5	9	$\pm$ 4	12	$\pm$ 1	24	$\pm$ 3
15	11	$\pm$ 4	106	$\pm$ 13	12	$\pm$ 0	14	$\pm$ 4	28	$\pm$ 1
50	13	$\pm$ 3	107	$\pm$ 13	17	$\pm$ 2	12	$\pm$ 4	21	$\pm$ 1
150	15	$\pm$ 2	109	$\pm$ 2	7	$\pm$ 0	14	$\pm$ 1	32	$\pm$ 1
500	21	$\pm$ 6	101	$\pm$ 1	11	$\pm$ 1	14	$\pm$ 0	22	$\pm$ 6
1500	20	$\pm$ 6	112	$\pm$ 17	14	$\pm$ 2	15	$\pm$ 5	24	$\pm$ 2
5000	14	$\pm$ 13	107	$\pm$ 6	13	$\pm$ 3	10	$\pm$ 0	24	$\pm$ 3
Positive	221	$\pm$ 13	346	$\pm$ 15	267	$\pm$ 17	2047	$\pm$ 40	331	$\pm$ 13

Activation Condition	Rat Liver S9									
Dose (μ g per plate)	TA98		TA100		TA1535		TA1537		WP2 uvrA	
Vehicle	21	$\pm$ 11	119	$\pm$ 13	10	$\pm$ 3	7	$\pm$ 4	40	$\pm$ 9
1.5	23	$\pm$ 1	135	$\pm$ 19	10	$\pm$ 7	9	$\pm$ 7	34	$\pm$ 8
5.0	26	$\pm$ 0	143	$\pm$ 7	11	$\pm$ 1	8	$\pm$ 3	40	$\pm$ 5
15	18	$\pm$ 1	167	$\pm$ 23	10	$\pm$ 4	8	$\pm$ 4	24	$\pm$ 4
50	19	$\pm$ 1	155	$\pm$ 1	10	$\pm$ 1	7	$\pm$ 2	37	$\pm$ 2
150	22	$\pm$ 3	144	$\pm$ 11	18	$\pm$ 6	9	$\pm$ 1	33	$\pm$ 6
500	20	$\pm$ 3	184	$\pm$ 34	14	$\pm$ 4	8	$\pm$ 2	32	$\pm$ 1
1500	21	$\pm$ 5	194	$\pm$ 31	17	$\pm$ 4	9	$\pm$ 2	24	$\pm$ 9
5000	24	$\pm$ 3	172	$\pm$ 50	15	$\pm$ 0	12	$\pm$ 4	23	$\pm$ 5
Positive	422	$\pm$ 37	412	$\pm$ 68	113	$\pm$ 9	68	$\pm$ 11	319	$\pm$ 6

Vehicle = Vehicle Control

Positive = Positive Control (50 μ L plating aliquot)

Plating aliquot = 50 μ L

Table 7-2 Results of the Bacterial Mutagenicity Assay (Confirmatory Mutagenicity Assay)

Test Article Id : H-28297
 Study Number : AC14XM.503.BTL
 Experiment No : B2 and B3
 Activation Condition : None

Dose (µg per plate)	TA98		TA100		TA1535		TA1537		WP2 uvrA	
Vehicle	18	± 3	142	± 19	11	± 3	14	± 4	22	± 1
50	16	± 3	144	± 12	10	± 5	10	± 2	22	± 3
150	16	± 3	134	± 7	13	± 2	13	± 5	23	± 4
500	18	± 1	149	± 7	15	± 3	10	± 2	22	± 1
1500	23	± 3	137	± 10	15	± 1	10	± 3	25	± 3
5000	17	± 4	138	± 28	16	± 5	11	± 4	21	± 3
Positive	261	± 65	593	± 59	455	± 28	1176	± 71	207	± 15

Activation Condition : Rat Liver S9

Dose (µg per plate)	TA98		TA100 ^a		TA1535		TA1537		WP2 uvrA	
Vehicle	19	± 9	109	± 16	13	± 3	10	± 3	29	± 0
50	31	± 2	118	± 9	14	± 4	9	± 6	23	± 4
150	22	± 5	120	± 6	12	± 1	10	± 3	31	± 2
500	23	± 7	111	± 2	11	± 2	9	± 3	26	± 6
1500	26	± 7	107	± 25	12	± 1	8	± 6	23	± 6
5000	26	± 3	120	± 20	11	± 3	12	± 3	25	± 1
Positive	700	± 533	261	± 7	98	± 25	54	± 20	242	± 38
Positive ^b			438	± 13						

Vehicle = Vehicle Control

Positive = Positive Control (50 µL plating aliquot)

Plating aliquot = 50 µL

^a Data from Experiment B3

^b 2-Aminoanthracene at 2.0µg per plate

7.1.2 Mouse Bone Marrow Erythrocyte Micronucleus Test

DuPont's EPA-rich triglyceride oil was tested in the mouse micronucleus assay (DuPont, 2009b). The assay was performed in two phases. The first phase, the dose range-finding phase (study), was designed to assess the toxicity of the test article and to set dose levels for the definitive micronucleus study. In the dose range-finding study, six groups of five male and five female mice were exposed to either vehicle control or EPA-rich triglyceride oil at 5%, 25%, 50%, 75% or 100% (neat test article). No mortality was observed in any treatment group. All animals dosed with the vehicle control and all female mice dosed with EPA-rich triglyceride oil in all treatment groups appeared normal. All male mice dosed with EPA-rich triglyceride oil had diarrhea. In the absence of mortality in the dose range-finding study, the high dose for the definitive micronucleus study was achieved by using 100% of EPA-rich triglyceride oil (undiluted, neat test article). Two additional dose levels at 50% and 25% of the neat EPA-rich triglyceride oil were also tested.

The second phase, the definitive micronucleus study, was designed to evaluate the potential of the test article to increase the incidence of micronucleated polychromatic erythrocytes in bone marrow of male and female ICR mice. The definitive micronucleus study consisted of four groups, each containing 10 male and 10 female mice. Mice in these groups were treated either with the vehicle control or with EPA-rich triglyceride oil at 25%, 50%, or 100%. Five mice/sex/treatment were euthanized 24 hours after dosing and the remaining five mice/sex/treatment were euthanized 48 hours after dosing. An additional group (group 5) of 5 male and 5 female mice was treated with CP simultaneously, and these animals were euthanized along with 24 hour treatment groups. At the time of euthanasia, femoral bone marrow was collected and bone marrow smears (slides) were prepared and stained with May-Gruenwald-Giemsa stain. Bone marrow cells [polychromatic erythrocytes (PCEs)] all 24-hour groups and the vehicle and high dose 48-hour groups were examined microscopically for the presence of micronuclei (micronucleated PCEs; MPCEs). A statistical analysis of data was performed using the Kastenbaum-Bowman Tables (binomial distribution, $p \leq 0.05$). The incidence of micronucleated PCEs and the ratio of PCEs to total erythrocytes (PCEs/ECs ratio) served as indication of test article clastogenicity and cytotoxicity, respectively.

Olive oil was used as the vehicle (negative) control in both studies as well as a diluent for the test article. In the definitive study, cyclophosphamide monohydrate (CP), at a dose of 50 mg/kg, was used as the positive control substance. In both phases of the study, test or control substances were administered at a dose volume of 20 mL/kg body weight by a single oral gavage. Animals were observed for signs of toxicity during the course of each phase of the study.

No mortality was observed in any treatment group. All animals dosed with the control substances (vehicle or positive) and all female mice dosed with EPA-rich triglyceride oil in all treatment groups appeared normal. All male mice dosed with EPA-rich triglyceride oil had diarrhea.

As shown in Table 7-3, no appreciable reductions in the ratio of polychromatic erythrocytes to total erythrocytes in the test article-treated groups relative to the respective vehicle control groups. No statistically significant increase in the incidence of micronucleated polychromatic erythrocytes in test article-treated groups relative to the respective vehicle control groups was observed in male or female mice at 24 or 48 hours after dose administration (Tables 7-4 and 7-5; $p > 0.05$). CP, the positive control, induced a statistically significant increase in the incidence of micronucleated PCEs ($p \leq 0.05$) in both male and female mice. The number of micronucleated PCEs in the vehicle control groups did not exceed the historical vehicle control range. Based upon this, all criteria for a valid test were met as specified in the protocol.

Under the conditions, a single oral administration of DuPont's EPA-rich triglyceride oil at doses up to and including 100% (neat test article) did not induce a significant increase in the incidence of micronucleated polychromatic erythrocytes in bone marrow of male or female ICR mice. Therefore, the EPA-rich triglyceride oil was concluded to be negative in the mouse micronucleus assay.

Table 7-3 Summary of Bone Marrow Micronucleus Analysis Following a Single Oral Administration of DuPont's EPA-Rich Triglyceride Oil in ICR Mice

Treatment (20 mL/kg)	Sex	Time (hr)	Number of Animals	PCE/Total Erythrocytes (Mean +/- SD)	Change from Control (%)	Number of MPCE/1000 PCE (Mean +/- SD)	Number of MPCE/PCE Scored
Olive Oil	M	24	5	0.448 ± 0.04	---	0.8 ± 0.45	8 / 10000
	F	24	5	0.478 ± 0.04	---	0.7 ± 0.27	7 / 10000
H-28297							
25%	M	24	5	0.470 ± 0.05	5	0.8 ± 0.57	8 / 10000
	F	24	5	0.474 ± 0.03	-1	0.8 ± 0.27	8 / 10000
50%	M	24	5	0.463 ± 0.04	3	0.7 ± 0.76	7 / 10000
	F	24	5	0.465 ± 0.04	-3	0.9 ± 0.22	9 / 10000
100%	M	24	5	0.481 ± 0.03	7	0.7 ± 0.27	7 / 10000
	F	24	5	0.478 ± 0.03	0	0.7 ± 0.57	7 / 10000
Cyclophosphamide							
50 mg/kg	M	24	5	0.461 ± 0.02	3	26.5 ± 4.51	*265 / 10000
	F	24	5	0.465 ± 0.03	-3	25.5 ± 2.78	*255 / 10000
Olive Oil	M	48	5	0.518 ± 0.01	---	0.7 ± 0.57	7 / 10000
	F	48	5	0.506 ± 0.01	---	0.6 ± 0.22	6 / 10000
H-28297							
100%	M	48	5	0.499 ± 0.02	-4	0.0 ± 0.00	0 / 10000
	F	48	5	0.513 ± 0.02	1	0.5 ± 0.50	5 / 10000

*Statistically significant increase, $p \leq 0.05$ (Kastenbaum-Bowman Tables)

Table 7-4 Induction of Micronucleated Polychromatic Erythrocytes in Bone Marrow Collected 24 Hours Following a Single Oral Administration of DuPont's EPA-Rich Triglyceride Oil in ICR Mice.

Treatment (20 mL/kg)	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE scored)
Olive Oil	M	1	0.474	3 / 2000
		2	0.425	1 / 2000
		3	0.487	2 / 2000
		4	0.398	1 / 2000
		5	0.458	1 / 2000
	F	6	0.523	2 / 2000
		7	0.439	2 / 2000
		8	0.473	1 / 2000
		9	0.516	1 / 2000
		10	0.438	1 / 2000
H-28297 25%	M	11	0.495	2 / 2000
		12	0.508	1 / 2000
		13	0.513	2 / 2000
		14	0.408	3 / 2000
		15	0.426	0 / 2000
	F	16	0.476	1 / 2000
		17	0.423	2 / 2000
		18	0.493	1 / 2000
		19	0.465	2 / 2000
		20	0.513	2 / 2000
50%	M	21	0.521	3 / 2000
		22	0.458	0 / 2000
		23	0.436	0 / 2000
		24	0.411	1 / 2000
		25	0.488	3 / 2000
	F	26	0.404	2 / 2000
		27	0.488	2 / 2000
		28	0.515	1 / 2000
		29	0.483	2 / 2000
		30	0.437	2 / 2000
100%	M	31	0.470	2 / 2000
		32	0.492	1 / 2000
		33	0.523	1 / 2000
		34	0.443	1 / 2000
		35	0.478	2 / 2000
	F	36	0.434	3 / 2000
		37	0.492	0 / 2000
		38	0.457	1 / 2000
		39	0.497	2 / 2000
		40	0.508	1 / 2000
Cyclophosphamide 50 mg/kg	M	41	0.441	58 / 2000
		42	0.455	54 / 2000
		43	0.479	63 / 2000
		44	0.493	51 / 2000
		45	0.439	39 / 2000
	F	46	0.477	58 / 2000
		47	0.499	56 / 2000
		48	0.425	47 / 2000
		49	0.460	46 / 2000
		50	0.466	48 / 2000

Table 7-5 Induction of Micronucleated Polychromatic Erythrocytes in Bone Marrow Collected 48 Hours Following a Single Oral Administration of DuPont's EPA-Rich Triglyceride Oil in ICR Mice.

Treatment (20 mL/kg)	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE scored)	
Olive Oil	M	51	0.523	2	/ 2000
		52	0.524	1	/ 2000
		53	0.515	3	/ 2000
		54	0.509	0	/ 2000
		55	0.519	1	/ 2000
	F	56	0.519	1	/ 2000
		57	0.483	1	/ 2000
		58	0.507	1	/ 2000
		59	0.516	1	/ 2000
		60	0.507	2	/ 2000
H-28297 100%	M	81	0.511	0	/ 2000
		82	0.462	0	/ 2000
		83	0.494	0	/ 2000
		84	0.510	0	/ 2000
		85	0.518	0	/ 2000
	F	86	0.532	1	/ 2000
		87	0.528	2	/ 2000
		88	0.482	0	/ 2000
		89	0.504	2	/ 2000
		90	0.521	0	/ 2000

7.1.3 *In Vitro* Mammalian Cell Gene Mutation Test

DuPont's EPA-rich triglyceride oil was tested in the L5178Y/TK^{+/−} Mouse Lymphoma Mutagenesis Assay in the absence and presence of Aroclor-induced rat liver S9 (DuPont, 2009c). The preliminary toxicity assay was used to establish the concentration range for the mutagenesis assay. The mutagenesis assay was used to evaluate the mutagenic potential of the test article.

In the preliminary toxicity assay, the maximum concentration of EPA-rich triglyceride oil in treatment medium was 5000 µg/mL in DMSO. Visible precipitate was present at concentrations ≥500 µg/mL in treatment medium. Selection of concentrations for the mutation assay was based on reduction of suspension growth relative to the solvent control and precipitation profile. Substantial toxicity (*i.e.*, suspension growth of ≤ 50% of the solvent control), was observed at 5000 µg/mL without activation with a 4-hour exposure and ≥1500 µg/mL with S9 activation with a 4-hour exposure and without activation with a 24-hour exposure.

Based on the results of the preliminary toxicity assay, the concentrations tested in the initial mutagenesis assay ranged from 5.0 to 750 µg/mL for both the non-activated and S9-activated cultures with a 4-hour exposure. Visible precipitate was present at concentrations ≥500 µg/mL in treatment medium. The concentrations chosen for cloning were 50, 100, 200, 350, and 500 µg/mL with and without S9 activation. No cloned cultures exhibited mutant frequencies ≥90 mutants per 10⁶ clonable cells over that of the solvent control. There was no concentration-related increase in mutant frequency (Tables 7-6 and Table 7-7).

Based on the results of the preliminary toxicity and initial mutation assays, the concentrations tested in the extended treatment assay ranged from 5.0 to 750 µg/mL for non-activated cultures with a 24-hour exposure. Visible precipitate was present at concentrations ≥500 µg/mL in treatment medium. The concentrations chosen for cloning were 50, 100, 200, 350, and 500 µg/mL. No cloned cultures exhibited mutant frequencies ≥90 mutants per 10⁶ clonable cells over that of the solvent control. As shown in Table 7-8, there was no concentration-related increase in mutant frequency.

The trifluorothymidine-resistant colonies for the positive (methyl methanesulfonate) and solvent control cultures from both assays were sized according to diameter over a range from approximately 0.2 to 1.1 mm. The colony sizing for the positive controls yielded the expected increase in small colonies (verifying the adequacy of the methods used to detect small colony mutants) and large colonies.

All criteria for a valid test were met. Under the conditions of this study, DuPont's EPA-rich triglyceride oil was concluded to be negative in the presence and absence of S9 metabolic activation in the L5178Y/TK^{+/−} Mouse Lymphoma Mutagenesis Assay.

Table 7-6 Data Summary for the Initial Assay (-S9, 4-hr exposure)

DOSE LEVEL (µg/mL)		PRECIP.	TOTAL SUSP. GROWTH	% SUSP. GROWTH	TFT COLONIES				VC COLONIES				TOTAL MUTANT FREQUENCY (PER 10 ⁶ CELLS)	INDUCED MUTANT FREQUENCY (PER 10 ⁶ CELLS)	% TOTAL GROWTH
					PLATE COUNTS				PLATE COUNTS						
					1	2	3	MEAN	1	2	3	MEAN			
SOLVENT 1			20.9	100	26	26	26	26	202	246	186	211	25	N/A	100
SOLVENT 2			20.7		*	31	19	25	140	140	140	140	36		
50	A		17.9	86	39	20	34	31	*	145	136	141	44	14	69
50	B		20.8	100	43	24	27	31	123	139	151	138	46	15	78
100	A		19.5	93	31	18	16	22	172	159	155	162	27	-3	86
100	B		19.3	93	22	26	*	24	182	155	162	166	29	-1	88
200	A		18.6	90	26	27	13	22	153	159	137	150	29	-1	76
200	B		19.3	92	15	19	18	17	131	152	182	155	22	-8	82
350	A		18.9	91	22	22	26	23	152	133	143	143	33	3	74
350	B		19.7	95	34	26	36	32	98	136	163	132	48	18	71
500	A	P	20.4	98	30	26	30	29	136	151	137	141	41	10	79
500	B	P	18.1	87	23	30	34	29	148	149	156	151	38	8	75
POSITIVE CONTROL: Methyl methanesulfonate (MMS) (µg/mL)															
20			8.8	42	35	42	22	33	26	7	13	15	430	400	4
15			12.2	58	73	79	78	77	35	42	40	39	393	363	13
MEAN SOLVENT TOTAL SUSPENSION GROWTH: 20.8															
MEAN SOLVENT CLONING EFFICIENCY: 88%															
MEAN SOLVENT MUTANT FREQUENCY: 30 (PER 10 ⁶ CELLS)															

Solvent = DMSO

A and B or 1 and 2 are duplicate cultures

* - Plate lost to contamination P = precipitating concentration

Table 7-7 Data Summary for the Initial Assay (+S9, 4-hr exposure)

DOSE LEVEL (µg/mL)		PRECIP.	TOTAL SUSP. GROWTH	% SUSP. GROWTH	TFT COLONIES				VC COLONIES				TOTAL MUTANT FREQUENCY (PER 10 ⁶ CELLS)	INDUCED MUTANT FREQUENCY (PER 10 ⁶ CELLS)	% TOTAL GROWTH
					PLATE COUNTS				PLATE COUNTS						
					1	2	3	MEAN	1	2	3	MEAN			
SOLVENT 1			16.8	100	20	28	18	22	167	147	118	144	31	N/A	100
SOLVENT 2			17.9		34	22	22	26	118	105	174	132	39		
50	A		15.3	88	30	27	31	29	131	133	118	127	46	11	81
50	B		18.6	107	30	22	30	27	131	105	133	123	44	10	96
100	A		16.9	98	16	23	13	17	141	160	162	154	22	-12	109
100	B		16.9	97	9	28	27	21	92	135	151	126	34	-1	89
200	A		15.3	88	19	39	23	27	136	135	132	134	40	5	86
200	B		16.9	98	16	27	34	26	132	143	164	146	35	0	103
350	A		16.8	97	20	23	23	22	132	145	141	139	32	-3	98
350	B		16.0	92	16	15	22	18	106	135	137	126	28	-7	84
500	A	P	12.3	71	23	19	15	19	166	144	160	157	24	-11	80
500	B	P	12.3	71	19	27	15	20	156	145	149	150	27	-8	77
POSITIVE CONTROL: 7,12-dimethylbenz(a)anthracene (DMBA) (µg/mL)															
1.25			1.4	10	82	129	129	113	31	35	34	33	680	645	2
1			3.1	18	98	123	144	122	63	57	48	56	435	400	7
MEAN SOLVENT TOTAL SUSPENSION GROWTH: 17.3															
MEAN SOLVENT CLONING EFFICIENCY: 69%															
MEAN SOLVENT MUTANT FREQUENCY: 35 (PER 10 ⁶ CELLS)															

Solvent = DMSO

A and B or 1 and 2 are duplicate cultures

P = precipitating concentration

Table 7-8 Data Summary for the Extended Treatment Assay (-S9, 24-hr exposure)

DOSE LEVEL (µg/mL)		PRECIP.	TOTAL SUSP. GROWTH	% SUSP. GROWTH	TFT COLONIES				VC COLONIES				TOTAL MUTANT FREQUENCY (PER 10 ⁶ CELLS)	INDUCED MUTANT FREQUENCY (PER 10 ⁶ CELLS)	% TOTAL GROWTH
					PLATE COUNTS				PLATE COUNTS						
					1	2	3	MEAN	1	2	3	MEAN			
SOLVENT 1			40.0	100	13	33	35	27	114	106	139	120	45	N/A	100
SOLVENT 2			39.6		24	26	*	25	139	151	****	145	34		
50	A		34.0	85	29	29	40	33	133	109	128	123	53	13	79
50	B		33.9	85	20	49	47	39	93	97	128	106	73	33	68
100	A		39.6	99	35	43	52	43	95	106	87	96	90	50	72
100	B		37.8	95	55	45	37	46	81	120	88	96	95	55	69
200	A		37.1	93	18	32	20	23	130	123	118	124	38	-2	87
200	B		34.6	87	18	49	49	39	115	97	139	117	66	26	77
350	A		35.0	88	22	13	27	21	104	123	144	124	33	-6	82
350	B		36.7	92	32	36	31	33	*	*	*				
500	A	P	39.1	98	27	14	37	26	124	120	86	110	47	7	81
500	B	P	39.3	99	19	40	23	27	109	144	*	127	43	3	94
POSITIVE CONTROL: Methyl methanesulfonate (MMS) (µg/mL)															
7.5			17.3	43	55	43	54	51	18	23	19	20	507	467	7
5			25.4	64	112	87	60	86	109	33	75	72	239	199	35
MEAN SOLVENT TOTAL SUSPENSION GROWTH: 39.8															
MEAN SOLVENT CLONING EFFICIENCY: 66%															
MEAN SOLVENT MUTANT FREQUENCY: 40 (PER 10 ⁶ CELLS)															

Solvent = DMSO A and B or 1 and 2 are duplicate cultures * - plate lost to contamination

P = precipitating concentration ****-Actual value cannot be determined based on hard evidence

7.2 Subchronic Toxicity

DuPont evaluated the potential repeated-dose toxicity of EPA-rich triglyceride oil produced from genetically modified *Yarrowia lipolytica* yeast, when administered by oral gavage to rats for approximately 28 days (DuPont, 2009d). This study was conducted in compliance with the U.S. FDA good laboratory practices and current OECD Good Laboratory Practices, as well as EEC and US EPA test guidelines. As shown in Table 7-9, fifteen CrI:CD(SD) rats per sex were administered EPA (neat or in olive oil) by intragastric intubation at a dose volume of 3 mL/kg body weight for at least 28 days in dosages of 325, 650, and 976 mg/kg b.w./day. Control animals received 3 mL olive oil vehicle while comparison oil rats were treated with 513 mg/kg/day of food-grade fish (sardine and anchovy) oil. The first 10 rats per group were designated for evaluation of repeated-dose toxicity and the remaining 5 rats per group were designated for evaluation of fatty acid profile in plasma and selected tissues. Tap water and feed were provided *ad libitum*. They were fed PMI® Nutrition International, LLC Certified Rodent LabDiet® 5002 *ad libitum* except when fasted. Body weights, food consumption, and detailed clinical observations were evaluated weekly and acute clinical observations were evaluated daily. Ophthalmological assessments were performed prior to the start of dosing and near the end of the exposure period.

Table 7-9 Study Design

Group		Number/Group ^a		Test Formulation	Nominal Dose Volume of H-28297 (mL/kg/day) ^b	Nominal EPA Dose (mg/kg) ^b	Group Designation
Male	Female	Male	Female				
1	1	15	15	Vehicle control (olive oil)	0	0	0 EPA Oil
2	2	15	15	H-28297: olive oil (1:2)	1	325	Low EPA Oil
3	3	15	15	H-28297: olive oil (2:1)	2	650	Medium EPA Oil
4	4	15	15	H-28297 (100%)	3	976	High EPA Oil
5	5	15	15	Fish oil (H-28728; reference oil)	0	513 ^c	Fish Oil

- a First surviving 10 rats/sex/group were designated for repeated-dose oral toxicity evaluation. The remaining rats were designated for biochemical evaluation.
- b Each animal was dosed with 3 mL/kg of the appropriate test formulation. The nominal dose of EPA was based on 34.6% EPA in H-28297 (EPA oil), w/w, and an estimated density of 0.94 mg/mL.
- c Based on a nominal density of 1 g/mL and 17.09% EPA in fish oil.

No test substance-related deaths occurred, and no clinical or ophthalmological observations were attributed to exposure to the test substance. No test substance-related effects on body weight, body weight gain, food consumption, or food efficiency were observed. There were no treatment-related changes in hematology (Tables 7-10 and 7-11). Total serum cholesterol was decreased in all EPA oil dose groups and in the fish oil group in males and females primarily due to decreases in the non-HDL fraction. However, this is an expected response to EPA exposure, and the decrease is of a magnitude considered to be potentially beneficial and comparable to that observed with other polyunsaturated fatty acids. No other test substance-related effects were observed on any other clinical chemistry parameters (Tables 7-12 and 7-13). Likewise, there were no changes in coagulation (Tables 7-14 and 7-15), or urinalysis parameters (Tables 7-16 and 7-17).

There were no test substance-related deaths or gross observations at necropsy. A test substance-related increase in liver weights was observed in the male and female High EPA and fish oil groups, and in males in the Medium EPA oil group. The increased liver weights were considered to be a nutritional pharmacological response, and they were not correlated with clinical or microscopic pathology evidence of liver toxicity. No other test substance-related effects on organ weights were observed.

Male rats given High EPA oil had a slightly greater incidence of thyroid follicular cell hypertrophy than the study control or historical control incidences. Thyroid follicular cell hypertrophy was not observed in any female rats on study. Since follicular cell hypertrophy is consistent with several different mechanisms of altered thyroid gland homeostasis, the specific cause and the biological significance of the hypertrophic response is not clear, based on the endpoints evaluated in this study. However, thyroid follicular cell hypertrophy was also observed at similar incidence in the male rats exposed to fish oil. Since this fish oil is generally recognized as safe (GRAS) by the U.S. FDA, this thyroid effect may represent an adaptive response to exposure to high levels of polyunsaturated fatty acids.

Under the conditions of this study, exposure to DuPont's EPA-rich triglyceride oil, dosed up to 100% (approximately 976 mg/kg/day of EPA), produced no adverse effects on in-life or clinical pathology parameters. As expected, all concentrations of EPA-rich triglyceride oil lowered cholesterol but to levels that may be associated with beneficial, and not adverse effects. Exposure to 100% or 67% (males only) EPA-rich triglyceride oil produced non-adverse increases in liver weights with no correlative clinical or microscopic pathology. Exposure to 100% EPA-rich triglyceride oil produced minimal to mild thyroid follicular cell hypertrophy in male rats, which was also observed at similar incidence in the male rats exposed to fish oil, a substance considered to be GRAS by the U.S. FDA.

Table 7-10

Summary of Hematology Values for Male Rats

	Group 1 0 mg/kg (n=9)	Group 2 325 mg/kg (n=9)	Group 3 650 mg/kg (n=8)	Group 4 976 mg/kg (n=10)	Group 5 Fish Oil (n=9)
RBC (x10 ⁶ /μL)	8.12 ± 0.26	8.05 ± 0.30	7.92 ± 0.32	8.03 ± 0.32	7.99 ± 0.26
HGB (g/dL)	15.4 ± 0.8	15.8 ± 0.4	15.3 ± 0.6	15.5 ± 0.6	15.6 ± 0.5
HCT (%)	47.9 ± 2.1	48.7 ± 0.9	47.2 ± 2.4	47.7 ± 1.9	47.6 ± 1.6
MCV (fL)	59.0 ± 1.6	60.7 ± 2.3	59.6 ± 1.0	59.4 ± 1.2	59.6 ± 1.5
MCH (pg)	18.9 ± 0.7	19.7 ± 0.7*	19.4 ± 0.3	19.3 ± 0.5	19.5 ± 0.6
MCHC (g/dL)	32.1 ± 0.6	32.4 ± 0.6	32.5 ± 0.6	32.6 ± 0.4	32.7 ± 0.7
RDW (%)	11.8 ± 0.5	11.9 ± 0.4	11.5 ± 0.4	11.8 ± 0.4	11.8 ± 0.4
ARET (x10 ³ /μL)	188.7 ± 20.1	171.3 ± 42.8	163.0 ± 14.2*	171.6 ± 35.5	183.7 ± 37.6
PLT (x10 ³ /μL) ¹	1143 ± 64	1175 ± 160	1203 ± 47	1097 ± 113	1036 ± 232
WBC (x10 ³ /μL)	14.32 ± 2.76	15.22 ± 4.00	14.14 ± 2.64	14.89 ± 3.21	15.87 ± 4.63
ANEU (x10 ³ /μL)	1.70 ± 0.55	1.47 ± 0.44	1.70 ± 0.82	1.77 ± 0.65	3.17 ± 3.98
ALYM (x10 ³ /μL)	11.94 ± 2.54	13.02 ± 3.93	11.67 ± 1.73	12.47 ± 3.00	11.95 ± 2.91
AMON (x10 ³ /μL)	0.37 ± 0.17	0.38 ± 0.12	0.42 ± 0.19	0.39 ± 0.15	0.36 ± 0.20
AEOS (x10 ³ /μL)	0.11 ± 0.05	0.13 ± 0.04	0.15 ± 0.15	0.11 ± 0.07	0.18 ± 0.12
ABAS (x10 ³ /μL)	0.06 ± 0.05	0.07 ± 0.03	0.06 ± 0.02	0.06 ± 0.03	0.05 ± 0.04
ALUC (x10 ³ /μL)	0.14 ± 0.08	0.15 ± 0.07	0.15 ± 0.12	0.11 ± 0.06	0.10 ± 0.07
AHSN (x10 ³ /μL)	-	-	-	-	0.50 ^a

1: Number of values included in calculation of platelets: Group 1, n=6; Group 2, n=5; Group 3, n=4; Group 4, n=6; Group 5, n=2).

A: Value from single animal. Due to lack of control value, statistical analyses were unable to be performed.

Data arranged as: Mean ± Standard deviation

* Statistically significant difference from control at p < 0.05 by Dunnett/Tamhane-Dunnett test.

@ Statistically significant difference from control at p < 0.05 by Dunn's test.

~ Due to lack of control values or variability among group means, statistical analyses were unable to be performed.

Table 7-11 Summary of Hematology Values for Female Rats

	Group 1 0 mg/kg (n=8)	Group 2 325 mg/kg (n=7)	Group 3 650 mg/kg (n=7)	Group 4 976 mg/kg (n=9)	Group 5 Fish Oil (n=10)
RBC (x10 ⁶ /μL)	8.17 ± 0.23	8.16 ± 0.28	8.22 ± 0.52	8.01 ± 0.31	8.15 ± 0.29
HGB (g/dL)	15.7 ± 0.5	15.9 ± 0.7	16.0 ± 0.7	15.6 ± 0.4	16.0 ± 0.6
HCT (%)	47.0 ± 1.2	47.8 ± 2.2	47.4 ± 2.2	46.5 ± 1.4	47.0 ± 1.6
MCV (fL)	57.5 ± 1.6	58.6 ± 1.7	57.7 ± 1.8	58.1 ± 1.3	57.7 ± 2.1
MCH (pg)	19.3 ± 0.6	19.5 ± 0.5	19.5 ± 0.7	19.4 ± 0.5	19.6 ± 0.7
MCHC (g/dL)	33.5 ± 0.8	33.3 ± 0.5	33.8 ± 0.6	33.4 ± 0.5	34.0 ± 0.5
RDW (%)	10.6 ± 0.2	10.5 ± 0.5	10.5 ± 0.2	11.0 ± 0.7	10.7 ± 0.3
ARET (x10 ³ /μL)	171.7 ± 37.0	162.0 ± 40.8	136.4 ± 26.2	148.6 ± 33.3	142.3 ± 30.7
PLT (x10 ³ /μL)	1066 ± 201	1203 ± 175	1071 ± 256	1079 ± 319	1036 ± 118
WBC (x10 ³ /μL)	13.12 ± 2.91	11.65 ± 2.57	15.42 ± 2.60	13.93 ± 4.92	13.14 ± 1.59
ANEU (x10 ³ /μL)	1.13 ± 0.32	1.20 ± 0.37	1.52 ± 0.47	1.18 ± 0.42	1.10 ± 0.33
ALYM (x10 ³ /μL)	11.31 ± 2.80	9.86 ± 2.14	13.16 ± 2.54	11.95 ± 4.32	11.39 ± 1.58
AMON (x10 ³ /μL)	0.31 ± 0.15	0.33 ± 0.15	0.37 ± 0.15	0.36 ± 0.14	0.32 ± 0.9
AEOS (x10 ³ /μL)	0.11 ± 0.05	0.09 ± 0.03	0.16 ± 0.9	0.18 ± 0.15	0.10 ± 0.03
ABAS (x10 ³ /μL)	0.07 ± 0.03	0.06 ± 0.04	0.07 ± 0.06	0.09 ± 0.04	0.07 ± 0.04
ALUC (x10 ³ /μL)	0.19 ± 0.15	0.11 ± 0.03	0.15 ± 0.10	0.17 ± 0.12	0.16 ± 0.13

1: Number of values included in calculation of platelets: Group 1, n=7; Group 2, n=3; Group 3, n=3; Group 4, n=5; Group 5, n=4).

A: Value from single animal. Due to lack of control value, statistical analyses were unable to be performed.

Data arranged as: Mean ± Standard deviation

* Statistically significant difference from control at p < 0.05 by Dunnett/Tamhane-Dunnett test.

@ Statistically significant difference from control at p < 0.05 by Dunn's test.

~ Due to lack of control values or variability among group means, statistical analyses were unable to be performed.

Table 7-12 Summary of Clinical Chemistry Values for Male Rats

	Group 1 0 mg/kg (n=10)	Group 2 325 mg/kg (n=10)	Group 3 650 mg/kg (n=10)	Group 4 976 mg/kg (n=10)	Group 5 Fish Oil (n=10)
AST (U/L)	78 ± 23	76 ± 17	69 ± 12	66± 5	73± 9
ALT (U/L)	29 ± 10	32 ± 11	29 ± 4	32± 4	34@± 5
SDH (U/L)	16.0 ± 2.7	16.8 ± 4.5	15.1 ± 4.3	14.0± 3.8	16.5± 3.0
ALKP (U/L)	221 ± 51	198 ± 32	245 ± 69	226± 55	244± 95
GGT (U/L)	0~+0	0~+0	0~+0	0~+0	0~+0
BILI (mg/dL)	0.16 ± 0.01	0.15± 0.02	0.15 ± 0.01	0.15± 0.02	0.17± 0.01
BUN (mg/dL)	11 ± 2	12 ± 2	13 ± 1	13± 1	14± 4
CREA (mg/dL)	0.31 ± 0.04	0.32 ± 0.05	0.30 ± 0.03	0.30± 0.03	0.32± 0.05
CHOL (mg/dL)	66 ± 16	53* ± 10	42* ± 7	42*± 7	48*± 9
TRIG (mg/dL)	74 ± 40	60 ± 30	61 ± 22	61± 22	73± 25
GLUC (mg/dL)	112 ± 45	110 ± 27	112 ± 19	112± 19	117± 39
TP (g/dL)	6.6 ± 0.2	6.6 ± 0.3	6.5 ± 0.3	6.5± 0.3	6.7± 0.3
ALB (g/dL)	3.5 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.5± 0.1	3.6± 0.1
GLOB (g/dL)	3.1 ± 0.2	3.1 ± 0.2	3.0 ± 0.2	3.0± 0.2	3.1± 0.2
CALC (mg/dL)	11.0± 0.5	10.9± 0.5	11.0± 0.3	10.8± 0.5	11.1± 0.3
IPHS (mg/dL)	9.2± 0.8	9.6± 1.6	9.6± 1.1	9.5± 0.7	9.5± 1.2
NA (mmol/L)	146.1± 1.1	145.9± 1.8	146.2± 2.2	145.3± 1.8	145.1± 1.7
K (mmol/L)	6.24± 0.43	6.29± 0.73	6.35± 0.64	6.65± 0.84	6.20± 0.54
Cl (mmol/L)	98.5± 2.6	99.6± 1.7	99.3± 2.7	98.6± 2.3	97.8± 2.0
HDL (mg/dL)	26± 6	23± 4	25± 5	21*± 3	23± 4
NHDL	40± 11	30*± 7	28*± 7	21*± 5	25*± 6
NEFA	0.81± 0.14	0.68± 0.16	0.67± 0.21	0.72± 0.20	0.76± 0.15

Data arranged as: Mean ± Standard deviation

* Statistically significant difference from control at p < 0.05 by Dunnett/Tamhane-Dunnett test.

@ Statistically significant difference from control at p < 0.05 by Dunn's test.

~ Due to lack of control values or variability among group means, statistical analyses were unable to be performed.

Table 7-13 Summary of Clinical Chemistry Values for Female Rats

	Group 1 0 mg/kg (n=10)	Group 2 325 mg/kg (n=10)	Group 3 650 mg/kg (n=10)	Group 4 976 mg/kg (n=10)	Group 5 Fish Oil (n=10)
AST (U/L)	73 ± 13	70 ± 9	68 ± 9	68 ± 3	69 ± 11
ALT (U/L)	26 ± 4	24 ± 4	24 ± 5	28 ± 3	26 ± 3
SDH (U/L)	14.7 ± 4.6	18.3 ± 3.6	17.7 ± 4.7	16.8 ± 1.5	16.2 ± 2.1
ALKP (U/L)	159 ± 57	144 ± 38	132 ± 45	168 ± 43	134 ± 27
GGT (U/L)	0~ ± 0	0~ ± 0	0~ ± 0	0~ ± 0	0~ ± 0
BILI (mg/dL)	0.19 ± 0.02	0.18 ± 0.03	0.18 ± 0.03	0.18 ± 0.02	0.18 ± 0.03
BUN (mg/dL)	15 ± 2	15 ± 2	15 ± 2	15 ± 2	14 ± 2
CREA (mg/dL)	0.31 ± 0.04	0.32 ± 0.04	33 ± 0.02	0.33 ± 0.02	0.32 ± 0.04
CHOL (mg/dL)	80 ± 16	65* ± 9	49* ± 12	49* ± 12	55* ± 9
TRIG (mg/dL)	36 ± 12	33 ± 6	38 ± 11	38 ± 11	34 ± 5
GLUC (mg/dL)	101 ± 9	99 ± 18	105 ± 12	105 ± 12	103 ± 6
TP (g/dL)	6.8 ± 0.3	6.9 ± 0.3	7.0 ± 0.4	7.0 ± 0.4	6.8 ± 0.2
ALB (g/dL)	3.8 ± 0.1	3.8 ± 0.2	3.9 ± 0.2	3.9 ± 0.2	3.8 ± 0.2
GLOB (g/dL)	3.1 ± 0.2	3.1 ± 0.2	3.1 ± 0.2	3.1 ± 0.2	3.0 ± 0.1
CALC (mg/dL)	10.7 ± 0.4	10.8 ± 0.3	10.7 ± 0.4	10.8 ± 0.3	10.7 ± 0.4
IPHS (mg/dL)	7.7 ± 1.3	8.0 ± 1.7	7.6 ± 0.8	7.7 ± 1.1	7.3 ± 0.6
NA (mmol/L)	144.3 ± 1.3	143.9 ± 1.8	143.2 ± 2.4	144.2 ± 2.3	144.3 ± 2.6
K (mmol/L)	5.5 ± 0.51	6.15 ± 0.95	5.76 ± 0.71	5.84 ± 0.41	5.64 ± 0.32
Cl (mmol/L)	100.9 ± 3.1	99.9 ± 1.3	98.7 ± 3.6	98.5 ± 2.1	99.8 ± 1.7
HDL (mg/dL)	30 ± 5	27 ± 3	24* ± 5	23* ± 5	25 ± 4
NHDL	50 ± 11	38* ± 7	30* ± 9	26* ± 7	30* ± 6
NEFA	0.74 ± 0.20	0.67 ± 0.18	0.75 ± 0.22	0.66 ± 0.18	0.74 ± 0.17

Data arranged as: Mean ± Standard deviation

* Statistically significant difference from control at p < 0.05 by Dunnett/Tamhane-Dunnett test.

@ Statistically significant difference from control at p < 0.05 by Dunn's test.

~ Due to lack of control values or variability among group means, statistical analyses were unable to be performed.

Table 7-14 Summary of Coagulation Values for Male Rats

	Group 1 0 mg/kg (n=10)	Group 2 325 mg/kg (n=9)	Group 3 650 mg/kg (n=10)	Group 4 976 mg/kg (n=10)	Group 5 Fish Oil (n=10)
PT (sec)	14.5 ± 1.0	14.7 ± 1.0	14.6 ± 1.0	14.6 ± 0.6	14.3 ± 1.0
APTT (sec)	14.5 ± 1.8	14.9 ± 1.7	14.5 ± 1.7	14.5 ± 1.6	14.4 ± 1.9

Data arranged as: Mean ± Standard deviation

* Statistically significant difference from control at p < 0.05 by Dunnett/Tamhane-Dunnett test.

@ Statistically significant difference from control at p < 0.05 by Dunn's test.

~ Due to lack of control values or variability among group means, statistical analyses were unable to be performed.

Table 7-15 Summary of Coagulation Values for Female Rats

	Group 1 0 mg/kg (n=9)	Group 2 325 mg/kg (n=9)	Group 3 650 mg/kg (n=9)	Group 4 976 mg/kg (n=9)	Group 5 Fish Oil (n=10)
PT (sec)	14.2 ± 1.0	14.5 ± 0.8	15.1 ± 0.6	14.1 ± 0.7	14.8 ± 0.9
APTT (sec)	14.2 ± 1.8	13.4 ± 1.9	14.1 ± 1.2	13.0 ± 1.1	13.7 ± 1.5

Data arranged as: Mean ± Standard deviation

* Statistically significant difference from control at p < 0.05 by Dunnett/Tamhane-Dunnett test.

@ Statistically significant difference from control at p < 0.05 by Dunn's test.

~ Due to lack of control values or variability among group means, statistical analyses were unable to be performed.

Table 7-16 Summary of Urinalysis Values for Male Rats

	Group 1 0 mg/kg (n=10)	Group 2 325 mg/kg (n=10)	Group 3 650 mg/kg (n=10)	Group 4 976 mg/kg (n=10)	Group 5 Fish Oil (n=10)
UVOL (mL)	10.8 ± 7.7	11.3 ± 5.2	9.2 ± 5.0	9.3 ± 4.0	14.6 ± 10.0
UOSM (mOsm/kg)	1033 ± 415	1047 ± 496	1413 ± 614	1288 ± 589	1004 ± 575
pH	6.5 ± 0.0	6.6 ± 0.3	6.5 ± 0.3	6.6 ± 0.3	6.8 ± 0.7
URO (EU/dL)	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.3	0.3 ± 0.3	0.4 ± 0.3
UMTP (mg/dL)	144 ± 77	183 ± 222	172 ± 97	129 ± 47	166 ± 99

Data arranged as: Mean ± Standard deviation

* Statistically significant difference from control at p < 0.05 by Dunnett/Tamhane-Dunnett test.

@ Statistically significant difference from control at p < 0.05 by Dunn's test.

~ Due to lack of control values or variability among group means, statistical analyses were unable to be performed.

Table 7-17 Summary of Urinalysis Values for Female Rats

	Group 1 0 mg/kg (n=10)	Group 2 325 mg/kg (n=10)	Group 3 650 mg/kg (n=10)	Group 4 976 mg/kg (n=10)	Group 5 Fish Oil (n=10)
UVOL (mL)	6.3 ± 2.8	5.3 ± 3.5	6.9 ± 5.6	8.4 ± 7.7	8.5 ± 5.0
UOSM (mOsm/kg)	1366 ± 578	1620 ± 676	1698 ± 1240	1804 ± 1809	1207 ± 1057
pH	6.6 ± 0.3	6.5 ± 0.4	6.6 ± 0.3	6.4 ± 0.3	6.7 ± 0.3
URO (EU/dL)	0.3 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	0.2 ± 0.0	0.2 ± 0.0
UMTP (mg/dL)	33 ± 11	42 ± 18	46 ± 32	49 ± 47	34 ± 26

Data arranged as: Mean ± Standard deviation

* Statistically significant difference from control at p < 0.05 by Dunnett/Tamhane-Dunnett test.

@ Statistically significant difference from control at p < 0.05 by Dunn's test.

~ Due to lack of control values or variability among group means, statistical analyses were unable to be performed.

8.0 SUMMARY AND CONCLUSION

The information presented herein shows that:

- (i) The chemical composition of DuPont's EPA-rich triglyceride oil is well characterized, and the manufacturing process yields a product demonstrated to reproducibly meet compositional specifications.
- (ii) Mutagenicity and genotoxicity assays (*i.e.*, reverse mutation, chromosomal aberration, and bone marrow micronucleus test), were conducted using DuPont's EPA-rich triglyceride oil. EPA-rich triglyceride oil was non-mutagenic and non-genotoxic in these assays.
- (iii) A repeated dose 28-day toxicity study was conducted with DuPont's EPA-rich triglyceride oil. Under the conditions of this study, exposure to EPA-rich triglyceride oil produced no adverse effects on in-life or clinical pathology parameters at doses of up to 976 mg/kg/day of EPA. As expected, all concentrations of EPA-rich triglyceride oil lowered cholesterol but to levels that may be associated with beneficial, and not adverse effects. Exposure to 100% or 67% (males only) EPA-rich triglyceride oil produced non-adverse increases in liver weights with no correlative clinical or microscopic pathology. Exposure to 100% EPA-rich triglyceride oil produced minimal to mild thyroid follicular cell hypertrophy in male rats, which was also observed at similar incidence in the male rats exposed to fish oil, a substance considered to be GRAS by the U.S. FDA.
- (iv) Omega-3 fatty acids, including EPA, have established physiological roles, and reports in the published scientific literature suggest that higher intakes of these fatty acids may afford health benefits

Based on the evidence provided above, including results of preclinical safety studies conducted with DuPont's EPA-rich triglyceride oil, the presence of an approximately 30-fold margin of exposure that exists between the NOAEL from the 28-day repeated dose toxicity study (976 mg/kg/day of EPA) and the maximum recommended dose to consumers (2 g EPA/day, or 33 mg/kg b.w./day for a 60 kg individual), and vast clinical experience with omega-3 fatty acids, including EPA, DuPont concludes that the chronic use of EPA-rich triglyceride oil in dietary supplements at a level of up to 2 g EPA/day will be reasonably expected to be safe.

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**DEPARTMENT OF HEALTH AND
HUMAN SERVICES****Food and Drug Administration****1 CFR Part 101**

[Docket No. 91N-0103]

RIN 0905-AB67

**Food Labeling: Health Claims and
Label Statements: Omega-3 Fatty
Acids and Coronary Heart Disease**AGENCY: Food and Drug Administration,
HHS.

ACTION: Final rule.

SUMMARY: The Food and Drug Administration (FDA) is announcing its decision not to authorize the use on the label or labeling of foods of health claims relating to an association between omega-3 fatty acids and coronary heart disease (CHD). The agency has determined, based on: (1) The totality of the publicly available scientific evidence; and (2) the agency's review of comments received in response to its November 27, 1991, proposed rule on omega-3 fatty acids and CHD, including scientific information included in those comments, that there is not significant scientific agreement among experts that such evidence supports a health claim for omega-3 fatty acids and CHD. Further, FDA has determined that the new information does not change the conclusions that the agency reached on the basis of the information reviewed in its proposal. Therefore, FDA has concluded that such a claim is not justified. This action is in response to provisions of the Nutrition Labeling and Education Act of 1990 (the 1990 amendments) that bear on health claims, and is developed in accordance with the final rule on general requirements for health claims, published elsewhere in this issue of the *Federal Register*.

EFFECTIVE DATE: May 8, 1993.

FOR FURTHER INFORMATION CONTACT: John C. Wallingford, Center for Food Safety and Applied Nutrition (HFS-465) Food and Drug Administration, 200 C St. SW., Washington, DC 20204, 202-205-5461.

SUPPLEMENTARY INFORMATION:

I. Background

In the *Federal Register* of November 27, 1991 (56 FR 60663), FDA proposed not to authorize a health claim relating to diets high in omega-3 fatty acids to reduced risk of heart disease. The proposed rule was issued in response to provisions of the 1990 amendments (Pub. L. 101-535) that bear on health claims and in accordance with the

proposed general requirements for health claims for food (November 27, 1991, 56 FR 60537). As amended in 1990, the Federal Food, Drug, and Cosmetic Act (the act) provides that a food is misbranded if it bears a claim that characterizes the relationship of a nutrient to a disease or health-related condition unless the claim is made in accordance with section 403(r)(3) or (r)(5)(D) of the act (21 U.S.C. 343(r)(3) or (r)(5)(D)).

Congress enacted the health claims provisions of the 1990 amendments to help U.S. consumers maintain good health through appropriate dietary patterns and to protect consumers from unfounded health claims. Section 3(b)(1)(A) of the 1990 amendments specifically requires the agency to determine whether health claims for 10 nutrient-disease relationships meet the requirements of section 403(r)(3) or (r)(5)(D) of the act. The relationship of omega-3 fatty acids and heart disease is one of the claims required to be evaluated. In the *Federal Register* of March 28, 1991 (56 FR 12932), FDA published a notice requesting scientific data and information on the 10 specific topic areas identified in the 1990 amendments. Relevant scientific studies and data received in response to this request were considered as part of the agency's review of the scientific literature on omega-3 fatty acids and CHD and were included in the proposed rule.

In addition, on January 30 and 31, 1992, FDA held public hearings on all aspects of the proposed rules (57 FR 239).

FDA requested in the *Federal Register* of November 27, 1991 (56 FR 60663), written comments in response to its proposed rule. FDA reviewed all of the comments it received, including new data submitted in the comments, and scientific articles referred to in the comments. FDA also reviewed additional scientific articles, reviews, and recommendations published from August 1991 through February 1992.

The Dietary Supplement Act of 1992 (DS Act) established a moratorium on the implementation of the 1990 amendments with respect to dietary supplements. The DS Act says that FDA can grant health claims for food, including dietary supplements, under section 403(r)(3)(B)(i) of the act. However, it may not act on such claims under section 403(r)(5)(D) of the act until it establishes a standard to implement that section of the act, which the DS Act says may not occur until December 1993. Section 3(b)(1)(A)(x) of the 1990 amendments directs the agency to evaluate the omega-3 fatty acids/CHD

claims based on the standard that FDA is establishing for determining the reliability of health claims under section 403(r)(5)(D) of the act. In the November 27, 1991, proposal on general requirements for health claims, FDA proposed to adopt the standard that the 1990 amendments provide for conventional foods, which is set forth in section 403(r)(3)(B)(i) of the act, as the standard for dietary supplements. Given this fact, and the fact that omega-3 fatty acids are found in numerous conventional foods as well as in dietary supplements, FDA broadened its inquiry to a determination as to whether it should grant a health claim on omega-3 fatty acids and CHD for any foods.

Because the DS Act provides that FDA may grant claims using the significant scientific agreement standard specified in section 403(r)(3)(B)(i) of the act, and given the breadth of FDA's November 1991, proposal on omega-3 fatty acids, FDA has decided to move forward to determine whether it can authorize a claim under section 403(r)(3)(B)(i) for omega-3 fatty acids and CHD.

However, this rule does not apply to dietary supplements. While a manufacturer of a dietary supplement can make a claim on omega-3 fatty acids and CHD without rendering its product misbranded under section 403(r)(1)(B) of the act, the manufacturer should assure itself that the making of the claim will not misbrand the product under section 403(a).

**II. Summary of Comments and the
Agency's Response**

FDA received 80 letters, each containing one or more comments, from consumers, health care professionals, universities and research institutes, health profession associations, consumer advocacy organizations, State and local governments, foreign governments, trade organizations, industry, and professional organizations. In addition to these comments, the agency also considered statements made on omega-3 fatty acids and CHD at the January 30 and 31, 1992, public hearings. Some of the comments agreed with one or more of the aspects of the proposed rule, without providing further grounds for support other than those provided by FDA in the preamble to the proposal. Other comments disagreed with one or more aspect of the proposal without providing specific grounds for the disagreement. A few comments addressed issues outside of the scope of this document and will not be discussed in this document. Most of the comments provided specific grounds in support of their positions concerning aspects of this health claim

as proposed. The agency has summarized and addressed the issues raised in the sections of this document that follow.

A. General Comments

1. Definition of omega-3 fatty acids and composition of omega-3 fatty acid supplements

1. One comment criticized the definition of omega-3 fatty acids used in the proposed rule, on the basis that omega-3 fatty acids were not distinguished from other polyunsaturated fatty acids (PUFA's).

In the proposed rule, FDA limited the term omega-3 fatty acids to eicosapentaenoic acid (EPA), 20 carbons, 5 double bonds) and docosahexaenoic acid (DHA), 22 carbons, 6 double bonds) (56 FR 60663 at 60664). FDA noted that most of the relevant research has used fish or fish oils rich in these two fatty acids.

FDA acknowledges that its statement Defining omega-3 fatty acids did not explicitly refer to omega-3 fatty acids. The sentence: "Their unique characteristic is the location of the first double bond, which occurs at the third carbon from the methyl (or omega) end of the fatty acid." (56 FR 60663 at 60664) was intended to refer to omega-3 fatty acids. This definition distinguishes omega-3 fatty acids from other PUFA's, which have their first, unsaturation at the sixth or ninth carbon from the omega end of the fatty acid.

2. One comment argued that the definition of omega-3 fatty acids should include land-based (primarily plant) omega-3 fatty acids (i.e., linolenic acid). (For the purposes of this document, the term linolenic acid is used to indicate the omega-3 fatty acid, alpha linolenic acid. In contrast, gamma linolenic acid has its first double bond at the sixth carbon from the omega end of the fatty acid, and is not an omega-3 fatty acid.)

FDA disagrees with this comment. FDA defined omega-3 fatty acids as EPA and DHA, primarily as a functional definition derived from the scientific literature. The hypothesis for a relationship between omega-3 fatty acids and CHD derived from correlations between low rates of CHD and high consumption of fish oils. Similarly, most of the intervention studies have used fish oil or fish as a source of EPA and DHA, not plant oils rich in linolenic acid. The comment provided no evidence that linolenic acid has biochemical effects comparable to EPA or DHA, nor has FDA found any evidence of a relationship between linolenic acid and CHD. Moreover, only a limited amount of linolenic acid is

converted in the body to EPA and DHA (Ref.100). Therefore, FDA believes it has represented the potential nutrient-disease relationship appropriately by limiting its attention to EPA and DHA.

3. Two comments stated that FDA's position on fish as opposed to the omega-3 fatty acids in fish was a tautology, because: "if polyunsaturated fatty acids have beneficial effects on CHD, and if fish oils are a member of this class of fatty acids, it should not be counted against their beneficial effects on CHD."

FDA disagrees with this comment. FDA considers the claim for omega-3 fatty acids to reflect the unique biochemistry of these fatty acids. In particular, the prevailing theory about the mode of action of omega-3 fatty acids is that they compete with omega-6 fatty acids (fatty acids with their first double bond at the sixth carbon from the methyl end, and which comprise the largest amount of dietary PUFA's). Thus, a clear separation of effects of omega-3 fatty acids from effects of other (primarily omega-6) PUFA's is needed to support a claim.

4. One comment stated that FDA did not consider the importance of the ratio of omega-3 fatty acids to arachidonic acid (AA), a 20 carbon omega-6 PUFA with four double bonds, and stated that evidence exists for a relationship between the saturated fat:unsaturated fat ratio in the diet and the omega-6:omega-3 fatty acid ratio in the diet and the risk of CHD.

FDA considers concerns about the ratio of AA to omega-3 fatty acids and the ratio of omega-6 fatty acids to omega-3 fatty acids to be reasonable in view of the competition between these classes of fatty acids in human biochemistry. FDA considered all types of foods and supplements used to provide omega-3 fatty acids in its evaluation of the claim. Although the AA content of the supplement was often reported, studies did not report data for total dietary AA. FDA is aware of only very limited data regarding the ratios of AA to omega-3 fatty acids, and of the omega-6 fatty acid:omega-3 fatty acid ratio in the diet and the risk of CHD. Therefore, it is not possible for FDA to draw any conclusions about these ratios and their possible modifying effects on the omega-3 fatty acids CHD health claim.

However, because the fish oils used contained high concentrations of omega-3 fatty acids, FDA believes that the amounts of fish oils supplemented to the various test diets would have affected the AA:omega-3 fatty acid ratio and the omega-6:omega-3 fatty acid ratio of the diets to some extent. FDA advises

that interested persons may petition FDA under § 101.70 (21 CFR 101.70) to issue a regulation regarding a health claim that relates these ratios to the risk of CHD.

5. Another comment pointed out that supplements used currently have contained various amounts of short- and long-chain omega-3 fatty acids and that many supplements also contain saturated fat. The comment stated that some of the discrepancies in reported findings may be due to the type of supplement used.

FDA agrees that numerous supplements varying in fatty acid composition have been used, and that the variation in the fatty acid composition of supplements may have influenced the outcome. FDA reexamined the studies cited in its proposal and the new data submissions for evidence that the nature of the supplement used was related to the outcome. However, the agency found that the same results are observed regardless of the source of omega-3 fatty acids. For example, in eight well-designed studies cited in the proposal on the total serum cholesterol response among normal subjects (Refs., 6, 9, 14, 49, 54, 73, 156, and 166), six different sources of omega-3 fatty acids were used: Salmon oil, SuperEPA, MaxEPA, a fish oil triglyceride, Promega, and mackerel paste. None of these supplements produced a change in total serum cholesterol. Similarly, four different sources of omega-3 fatty acids (fresh water fish, salmon oil, purified EPA, MaxEPA) were shown in seven Well-designed studies to reduce platelet aggregation in normal subjects (Refs. 2, 6, 24, 54, 96, 143, and 166).

FDA did note that some differences in response have been produced by supplements that vary in ratio of EPA to DHA. For example, one fish oil (pollock oil) with a high EPA:DHA ratio increased low-density lipoprotein (LDL) cholesterol, LDL triglyceride and apoprotein B (apoB) (a protein component of LDL) in comparison to a butter-rich diet, but two fish oils with a low EPA:DHA ratio (tuna oil, salmon oil with added palmitic acid) reduced apoB and LDL cholesterol, and increased LDL triglyceride to a smaller extent than the pollock oil in comparison to the butter-rich diet (Ref. 17). However, the effects of the two major omega-3 fatty acids have not yet been systematically investigated. FDA recognizes that purified EPA and DHA are now available for research; such supplements will enable the study of the individual effects of these fatty acids.

6. One comment stated that conservation of omega-3 fatty acids in

the body calls into question the importance of the amounts of omega-3 fatty acids used in scientific studies.

However, the comment did not suggest any alternate method to describe intake.

FDA recognizes that fish is not ordinarily consumed daily. However, the 1990 amendments require that health claims on foods be stated in such a way as to enable the public to understand the relative significance of such information in the context of a total daily diet (section 403(r)(3)(B)(iii) of the act). Thus, a reasonable estimate of daily dietary intake of omega-3 fatty acids is needed when assessing the relationship between omega-3 fatty acids and the risk of CHD. Most of the studies reviewed by the agency used daily supplementation with a known amount of omega-3 fatty acids, but others estimated intake of omega-3 fatty acids from foods consumed in the daily diet. Both types of intake estimates are important. Daily supplementation is useful to relate changes to a carefully controlled amount of omega-3 fatty acids. The average daily intake of omega-3 fatty acids in nonintervention studies provides a basis upon which to determine whether the amounts of omega-3 fatty acids fed in supplementation studies are reasonable in the context of the total daily diet

Criteria used in evaluating studies

In the proposed rule, FDA listed some of the criteria used in evaluating epidemiological studies on the relationship of omega-3 fatty acids to CHD: (1) The reliability and accuracy of the methods used in food intake analysis and measurements of disease endpoints, (2) the choice of control subjects, (3) the representativeness of the subjects, (4) the control of confounding factors in data analysis, (5) the potential for misclassification of individuals with regard to dietary exposure or disease endpoints, (6) the presence of bias, and (7) the degree of compliance and how compliance was assessed (56 FR 60667).

However, FDA stated that it considered randomized, double-blind, placebo-controlled trials to be more valuable than other types of human studies because they were less susceptible to bias, and because they allowed inference about the specific effects of omega-3 fatty acids. Studies in which the endpoint was CHD, by definition, provide the most persuasive type of evidence, but studies measuring CHD to date have not provided the specificity to show that the observed effects were due to omega-3 fatty acids.

7. Some comments expressed the concern that it was unlikely that

additional clinical trials will be done due to their expense, and that, therefore, FDA should rely more heavily on epidemiologic studies, animal studies, and biochemical and physiological interventions that suggest an effect of omega-3 fatty acids on risk of CHD.

FDA has no basis upon which to agree or disagree with the comments' assertion that further clinical trials are unlikely. FDA disagrees that its emphasis on clinical trials was misplaced. Because the 1990 amendments addressed nutrient-disease relationships, FDA considered human studies that used CHD as the endpoint to be the most directly relevant studies, although these studies do not demonstrate that the effects are specifically due to omega-3 fatty acids. Human studies in which a surrogate marker for CHD risk was measured as the endpoint of the treatment were also considered carefully. The advantage of these studies is that they are able to demonstrate specificity of the effects due to omega-3 fatty acids. However, the relationships between many of these surrogate markers and risk of CHD are not well established, making it difficult to relate changes in these endpoints brought about by omega-3 fatty acids to the risk of disease. Biologic markers can serve as markers of a developing disease, but the relevance of such evidence depends directly on the strength of the association between the marker and the disease (Ref. 115).

FDA agrees that there are considerable additional data in animal studies, in vitro studies, and biochemical and physiological interventions regarding the effects of omega-3 fatty acids. However, it is not clear that the results of such studies are relevant to the risk of human disease. Thus, FDA believes that these other types of data are of secondary importance compared to clinical data that measure either CHD per se or established surrogate markers for CHD.

However, in response to the comments, FDA has provided a more thorough description of animal and in vitro studies that suggest a role for omega-3 fatty acids in reducing the risk of CHD, particularly with respect to the effects of omega-3 fatty acids on the development of atherosclerosis and with respect to the responsiveness of blood vessels to ischemia (see comments 38 and 49 and section II.C.3.a. of this document).

8. Many comments stated that the agency's position on omega-3 fatty acid and CHD was inconsistent with its position on other health claims, and argued that for each of the four claims proposed to be allowed by FDA, the

data were no stronger than the data supporting the link between omega-3 fatty acids and CHD. The comments asserted that, by basing its decision on the relationship between the nutrient and a surrogate marker for the disease, or for a susceptible subpopulation, FDA held other claims to a less restrictive standard. One comment stated: "The FDA statement is internally consistent in denying health claims for omega-3 fatty acids, but this is only in the context of holding these food components to essentially impossible standards not required for other, allowable, claims."

Specific comparisons were made to the proposed claims on fat and CHD, fat and cancer, calcium and osteoporosis, and sodium and hypertension. Other comments indicated that qualified claims, such as that for calcium and osteoporosis, were appropriate models for the claim relating omega-3 fatty acids to CHD.

FDA disagrees with these comments. FDA believes that for these other claims there is significant scientific agreement among qualified experts regarding the relationship between the nutrient and the disease, whereas there is not such agreement regarding the relationship between omega-3 fatty acids and CHD, or between omega-3 fatty acids and agreed surrogate markers for risk of CHD. For example, based on the totality of the publicly available scientific evidence, FDA determined that there is significant scientific agreement about the role of calcium in maintaining bone mineral density (the relationship of the nutrient to the intermediate marker for the disease), and about the relationship between peak (maximal) bone mass and the risk of developing osteoporosis and related bone fractures later in life (the relationship between the intermediate marker and the disease itself) (see 56 FR 60689; see also the final rule on calcium and osteoporosis published elsewhere in this issue of the **Federal Register**). Similarly, FDA relied on a long history of Federal Government and other consensus statements to conclude that there is significant scientific agreement about the role of sodium as a causal factor in hypertension for a segment of the population. (See 56 FR 60825; see also the final rule on sodium and hypertension, published elsewhere in this issue of the **Federal Register**.) FDA also recognized the history of significant scientific agreement about the relationships between fat and cancer and between fat and CHD evidenced by statements in reports issued by Federal Government and other authoritative bodies. (See 56 FR 60764, 56 FR 60726; see also final rules on fat and cancer and

fat and CHD, published elsewhere in this issue of the **Federal Register**.)

Thus, these other nutrient-disease relationships have a history of being recognized in Federal Government and authoritative reports, indicating significant scientific agreement, whereas the relationship between omega-3 fatty acids and CHD has not been so recognized. For two of these other nutrient-disease relationships, the data relate to the disease itself, rather than to markers for the disease. In the other two, calcium and osteoporosis and fat and CHD, there is significant scientific agreement that the dietary factors are related to surrogate markers for the diseases, and that the surrogate markers are related to the diseases.

There is significant scientific agreement that serum cholesterol and blood pressure are risk factors for CHD, as indicated by the emphasis on these factors in Federal Government and other authoritative documents (Refs. 34 through 36, 100, 115, and 169). Data regarding the effects of omega-3 fatty acids on these endpoints have been carefully reviewed. However, the other endpoints measured in studies of the effects of omega-3 fatty acids, e.g., in vitro platelet aggregation, various growth factors, fibrinogen, have not achieved the same extent of scientific agreement.

Where authorized health claims include qualifications, the qualifications are intended to assure that the wording of allowed claims reflects those particular aspects of the substance-disease relationship for which there is significant scientific agreement, not to qualify the extent of agreement.

9. Some comments stated that FDA relied heavily on material published in the National Academy of Sciences 1989 report, "Diet and Health: Implications for Reducing Chronic Disease Risk" (Ref. 115) and the Surgeon General's 1988 report (Ref. 34), and did not place enough emphasis on information published since that time.

FDA acknowledges that the two reports in question were important to its assessment of the scientific evidence. However, the agency does not agree that it failed to give appropriate weight to subsequently published research. The 1990 amendments required the agency to consider the totality of publicly available scientific evidence in assessing nutrient-disease relationships. Given the time constraints imposed by the 1990 amendments for developing and publishing proposed regulations, FDA depended on Federal Government reports and reports of authoritative bodies (e.g. the National Academy of Sciences) for assessment of the scientific

evidence published before 1988. The reports were also used as a way of determining whether there was significant scientific agreement among qualified experts that the evidence supports a relationship between omega-3 fatty acids and CHD. The agency's reliance on these reports is consistent with the 1990 amendments, which require the agency to consider reports from authoritative scientific bodies of the United States in assessing health claim petitions and to justify any decision rejecting the conclusions of such reports (section 403(r)(4)(C) of the act).

Recognizing, however, that considerable research had been published since these reports, and that these reports had not been updated, FDA also reviewed the available studies on humans published since 1988. FDA relied on its own review of individual studies rather than review articles, because review articles generally reflect the bias of the author and may not consider the totality of the evidence. FDA focused its independent review on primary papers published between January 1988 and August 1991. Surveys and cross-sectional or prospective studies that were published before 1988 and used to generate the hypothesis of a relationship between omega-3 fatty acids and CHD were also reexamined. Thus, by utilizing the two reports in question, supplemented with an independent review of the subsequently published research, FDA was able to assess the totality of the scientific evidence on omega-3 fatty acids and CHD in compliance with the statutory standard.

10. One comment suggested that FDA was inconsistent with the conclusions of the major reviews of this area, published after the Federal Government and other comprehensive reports. They stated that of the nine major reviews (excluding Kinsella, and Connor and Connor), eight concluded that omega-3 fatty acids played a beneficial role with factors affecting heart disease.

Although FDA did not rely on review articles to assess the strength of association between omega-3 fatty acids and CHD, each review was read, and the agency interprets these reviews as supporting the hypothesis in concept. However, each review contained reservations about the extent to which the relationship between omega-3 fatty acids and CHD was established. The cautionary statements suggest general agreement that the area of omega-3 fatty acids and CHD holds promise for further research along a number of lines, but that, at present, there are not sufficient data to have certainty about the

relationship between omega-3 fatty acids and CHD. Placed in chronological order, the concluding sections from the cited review articles exemplify the lack of certainty as to the relationship between omega-3 fatty acids and risk of CHD.

The review of the relationship between omega-3 fatty acids and CHD by Leaf and Weber (Ref. 91) was considered in the National Academy of Sciences' "Diet and Health: Implications for Reducing Chronic Disease Risk" report (Ref. 115). FDA elected to include the Leaf and Weber review in its citations because it covered, in the most comprehensive manner of all available reviews, the state of scientific knowledge about omega-3 fatty acids in CHD at the time the Federal Government and other comprehensive reviews were published. Leaf and Weber wrote: "Despite claims that n-3 fatty acids can help prevent atherosclerosis, recommendations to the public on diet have been conservative; people have been advised to increase their consumption of fish by replacing two or three meals a week containing red meat with meals containing fish." Their concluding sentence was: "If prospective double-blind, placebo-controlled clinical trials were to show that n-3 fatty acids helped to prevent atherosclerosis, these agents apparently would represent one of the most benign interventions in our pharmacopeia." (Emphasis added.)

Bonaa (Ref. 10) wrote in his conclusion that the data on blood pressure:

*** provide some support for the hypothesis that dietary marine lipids influence blood pressure in man. Supplementation of n-3 PUFAs [polyunsaturated fatty acids] to Western diets consistently lowered systolic blood pressure, while results for diastolic blood pressure were conflicting ***. There is no evidence of any substantial hypotensive response to marine lipids and further studies should be designed to detect small effects.

Lands (Ref. 89) did not review the relationship between omega-3 fatty acids and any specific disease, but presented the hypothesis that the balance of omega-3 and omega-6 fatty acids in the diet may be related to diseases associated with overproduction of eicosanoids from AA. He indicates in the introduction that, "We are now in an uncertain time of evaluating the benefits and risks of dietary n-6 and n-6 polyunsaturated fats."

Weber (Ref. 161) concluded:

The promise of n-3 fatty acids deduced from biochemical and functional effects will have to be evaluated in ongoing and future carefully designed and conducted studies. So

far, published data of controlled clinical trials incorporating clinical endpoints after n-3 PUFAs are available only in abstract form. Therefore, the gap between biochemical and functional effects of dietary fatty acids is assumed to be of clinical benefit in the prevention of atherothrombotic and allergic/inflammatory disorders is only beginning to be closed. (Emphasis added.)

Connor and Connor (Ref. 21) wrote in their summary:

The exact place of omega-3 fatty acids from fish and fish oil remains to be defined. However, this much seems certain. Fish provides an excellent substitute for meat in the diet. Fish is lower in fat, especially saturated fat, and contains the omega-3 fatty acids. Fish oil may have promise as a therapeutic agent in certain hyperlipidemic states, especially the chylomicronemia of type V hypolipidemia. Fish oil has logical and well-defined antithrombotic and antiatherosclerotic activities since it depresses thromboxane A₂ production and inhibits cellular proliferation responsible for the progression of atherosclerosis. As the years pass and more experiments are reported, it seems reasonable to place the omega-3 fatty acids from fish oil in a prominent position for specific hypolipidemic, antithrombotic and antiatherosclerotic activity.

Kinsella et al. (Ref. 82) wrote:

The cumulative findings concerning fish oils suggest that further amelioration of coronary heart disease may be feasible by dietary manipulation and by optimizing the intake of n-6 and n-3 PUFAs, not only to reduce plasma lipids but to ensure balanced eicosanoid metabolism—a prospect that deserves more research * * *. Overall, in view of the prevalence of coronary heart disease, consumption of n-3 PUFA oils should be considered as a useful complementary option for the amelioration of coronary vascular diseases.

Knapp (Ref. 84) introduced his paper stating: "The role of dietary polyunsaturated fats in the prevention of human vascular disease *has not been defined*, but population and intervention studies have *suggested* that w-3 fatty acids (FAs) from marine lipids *may have* a number of potentially beneficial effects." (Emphasis added.) And in conclusion he wrote: "The *proof of our hypotheses* must be derived from increasingly ambitious clinical trials, which assess the *potential* benefits of dietary polyunsaturates in particular clinical settings, the recent demonstration that three helpings of oily fish per week prolongs survival after MI (Ref. 16) is an example of this." (Emphasis added.)

Nestel (Ref. 111) concluded: "More basic understanding of the actions of fish oils is necessary before fish oils can be recommended widely to the public."

Nordoy and Goodnight (Ref. 112) cautioned that until additional data

become available, "clinicians should be advised to follow the dietary recommendations of the National Cholesterol Education Program's expert panel," which is silent on omega-3 fatty adds and limits the total : polyunsaturated fat to 10 percent of calories. These reviewers added their own recommendation that omega-6/omega-3 ratio of the PUFA's be approximately 3/1, with the omega-3 fatty acids from marine sources.

Weber and Leaf (Ref. 162) stated:

Despite all the laboratory, human, animal, and epidemiologic studies suggesting an anti-atheromatous action of w-3 fatty acids, we have been lacking adequate clinical trials which will determine in prospective, placebo-controlled, randomized studies, whether all the above experimental and epidemiologic evidence adds up to a demonstrable effect of fish oils to prevent atherosclerosis, e.g., coronary heart disease in humans at high risk for heart attacks.

The Burr paper (Ref. 16) was described in Weber and Leaf's review, and thus was considered in the above summary statement.

In summary, these reviews indicate that what is agreed is that there is a plausible biochemical basis for a relationship between omega-3 fatty acids and CHD, and that there are some data supporting some of the hypothesized mechanisms by which omega-3 fatty acids might be related to CHD. What is not agreed, as indicated by the cautious tones of these concluding statements, is that such a relationship already has been established by the evidence.

11. A concern raised by many comments was that FDA's conclusions were different from the conclusions reached in the report from the Life Sciences Research Office (LSRO) of the Federation of American Societies for Experimental Biology (Ref. 100), the most recent comprehensive review, and that FDA did not explain why it reached a different conclusion from that reached in the LSRO report.

The LSRO report was contracted for by FDA as an independent review of the scientific evidence about the relationship between omega-3 fatty acids and CHD. A draft of the tentative final report was received immediately prior to the publication of the proposed rule. Thus, there was insufficient time for the agency to prepare a detailed discussion of the report. The final report was submitted to FDA as a comment to the proposal. The LSRO report's conclusions on hypertension, thrombosis, the development of atherosclerotic plaque and intimal hyperplasia, plasma lipids and lipoproteins, diabetic and prediabetic

patients, and epidemiologic observations are grouped with other comments on these topics and discussed in this document.

12. One comment considered FDA's caution against extrapolation of results from studies conducted in at-risk populations to the general population to be questionable, and possibly biased against hypertensives. The comment stated that the health claim should be allowed, based on data showing that omega-3 fatty acids reduce blood pressure among hypertensives.

FDA disagrees with this comment. FDA stated that, although it considered studies in the healthy population to be the most relevant, it also considered studies in a subpopulation with CHD or risk factors for CHD, in part because high risk populations may be more sensitive to showing a nutrient-disease relationship than the general population (56 FR 60663 at 60667). FDA stated that it extrapolated positive results from at-risk populations cautiously, and that comparable findings in the general population were needed to support a health claim.

13. Two comments discussed FDA's criteria for weighing various types of data. One comment stated that epidemiologic data are the "most significant class of evidence," and that FDA should give priority to various types of data in the same order that various types of data were reviewed in the proposal. One comment stated that FDA should not have considered epidemiological studies separately from clinical trials.

FDA considered the totality of publicly available scientific evidence in its assessment of the relationship of omega-3 fatty acids to CHD. However, some types of evidence were weighted more heavily than others because they were more useful in establishing whether or not the scientific basis of the claim was valid. In particular, the agency was concerned that both the substance (omega-3 fatty acids) and the disease (CHD) be carefully characterized. FDA also considered it important that the amount of omega-3 fatty acids tested was reasonably related to normal dietary intake, and that the findings apply to the general population. FDA agrees that epidemiologic studies in which the endpoint was CHD provide persuasive evidence for a relationship between fish consumption and CHD, but these studies did not provide the specificity to show that the observed effects were due to omega-3 fatty acids. Intervention trials using fish oil supplements often showed that the effects were specific to omega-3 fatty acids (by controlling with

other types of fatty acids) but typically did not measure the primary endpoint, CHD. Thus, these different types of data complement each other and must be considered together in assessing the totality of the scientific evidence.

14. One comment offered the services of the International Society for the Study of Fatty Acids and Lipids for the evaluation of the relationship between omega-3 fatty acids and CHD.

FDA appreciates this offer. In the final rule on general requirements for health claims published elsewhere in this issue of the *Federal Register*, FDA advises that it welcomes the input of any professional organization that can provide expertise in reviewing data and in developing a thoughtful and well-organized petition for a health claim on a particular topic. In fact, FDA has added to § 101.70(b) the provision that information submitted with petitions may include any findings, along with the basis of the findings, of an outside panel with expertise in the subject area at issue. FDA, however, retains the authority to review such petitions and, through rulemaking, to decide whether or not to authorize the claim.

15. Two comments stated that it was contradictory for the U.S. Government to contract for research on the omega-3 fatty acids through the biologic test materials program but not to allow a health claim. Another comment pointed out that the Food and Agriculture Organization and the World Health Organization support research on omega-3 fatty acids. Other comments stated that the tone of FDA's proposed rule was unduly negative and that, by taking such a position, FDA may retard further research.

FDA disagrees that Federal Government sponsorship of a program to provide test materials for research on the effects of omega-3 fatty acids and the denial of the omega-3 fatty acid-CHD health claim are contradictory actions. The purpose of the biologic test materials program is to develop, and standardize a source of omega-3 fatty acids and enable carefully controlled research on the effects of particular omega-3 fatty acids. In the proposed rule, FDA's intent was to examine the total available scientific evidence, some of which was generated using omega-3 fatty acids from the biologic materials test program, and to state its conclusions about the relationship between omega-3 fatty acids and CHD.

In its proposal and in this final rule, FDA has identified a number of areas where agreement is lacking that an observed effect of omega-3 fatty acids is related to the risk of CHD, or where there are ambiguities in the data that

may be resolved by further research. Thus, FDA's analysis should provide guidance for additional research rather than inhibit it.

B. Relationship Between Omega-3 Fatty Acids and CHD

In the proposed rule, FDA tentatively concluded that the totality of the scientific evidence does not provide a basis upon which to authorize a claim that omega-3 fatty acids are associated with the risk of CHD (56 FR 60663). FDA noted that.

the epidemiological research on this topic revealed that the available studies applied only to the consumption of fish, which contain omega-3 fatty acids, and *** it was not possible to ascribe any effects specifically to the omega-3 fatty acids. Examination of data from clinical studies revealed that the effects on blood lipids of fish oils containing omega-3 fatty acids were primarily a reduction of blood triglycerides, a blood lipid variable not considered to be an independent risk factor for CHD, but they had no effect on serum cholesterol, low-density lipoprotein (LDL) cholesterol, or high density lipoprotein (HDL) cholesterol, the blood lipid variables most closely associated with risk of CHD. The scientific data are ambiguous on the effects of omega-3 fatty acids on blood pressure and other risk factors for CHD. (56 FR 60663.)

A number of comments supported the agency's position on this health claim, but without any specific reasons for that support. One comment agreed with the agency's position in principle, but contested the agency's interpretation of the scientific information in some areas. Other comments disagreed with the agency's review of the scientific information and its conclusion regarding the strength of the evidence supporting the proposed health claim. Specific comments are summarized below.

1. Epidemiologic evidence

In the proposed rule, FDA reviewed correlational and cross-sectional studies, prospective studies, and intervention studies available since 1988. (See 56 FR 60663 at 60667 through 60668). Except for the intervention studies (which were typically clinical trials) these studies used fish as a source of omega-3 fatty acids. FDA concluded that those studies that used fish as the source of omega-3 fatty acids were: "ambiguous, because they are not capable of distinguishing the effects that are specific to omega-3 fatty acids from those that are related to fish consumption." (56 FR 60663 at 60668.)

16. A number of comments considered the evidence from epidemiologic studies that relates the

consumption of fish inversely to CHD to be sufficient to support a health claim, but did not supply any new information or arguments to support their position.

FDA disagrees with the comments FDA found that:

Only a few studies found an association between, fish intake and CHD, while others have found no association. Thus there was not consistency of findings. None of the studies that reported a relationship distinguished fish consumption from other factors associated with fish consumption, and therefore they did not demonstrate specificity. Even in those studies reporting a relationship between fish consumption and CHD, it was not clear that the effects were because of the omega-3 fatty acids in fish. Also, the omega-3 fatty acid content of the fish diet associated with reduced CHD was so low that the importance of omega-3 fatty acids is questionable ***

(56 FR 60663 at G0672.)

17. One comment described the results of the Dolecek and Grandits analysis of multiple risk factor intervention trial (MKFIT) data (Ref. 38) as indicating a greater protective effect against CHD due to consumption of 0.6 gram (g) omega-3 fatty acids than all other conventional efforts combined (reducing saturated fat, cholesterol, cigarette smoking, and hypertension).

FDA agrees with this comment that the association between omega-3 fatty acid consumption and CHD mortality reported in this study has the potential to make a very important public health impact. Notably, the results were obtained on data adjusted for age, race, smoking at entry to the study, diastolic blood pressure, and high-density lipoprotein (HDL) and LDL concentrations. Furthermore, the omega-3 fatty acids were obtained in the normal diet, providing evidence that the amount of omega-3 fatty acids consumed in a normal dietary intake is sufficient for the effect.

The researchers' adjustments for lipoprotein measurements should control for some other dietary variables that have been associated with CHD through their effects on these lipoproteins, e.g., saturated fat, but other dietary variables associated with CHD were not controlled, e.g., alcohol. The association between omega-3 fatty acid consumption and CHD mortality described in this study is among the most provocative findings to date in this area, and merits additional study using a design that will document that the active dietary component' is or is not the omega-3 fatty acids (i.e., specificity of the effect).

18. One comment pointed out that the Burr paper (Ref. 16) deserved close consideration, because, in contrast to

trials on lipid lowering drugs, it showed that consumption of fish containing omega-3 fatty acids or dietary supplements of omega-3 fatty acids may reduce the risk of heart disease. One comment stated that it considered the Burr paper to be a positive finding, but gave no reason for this conclusion. The LSRO final report, submitted as a comment, also recognized the Burr paper as a very important trial. LSRO pointed out that, although separate results were not shown for those consuming fatty fish and those consuming supplemental fish oil, the results were dramatic, especially since all-cause mortality was reduced, in contrast to results from trials of plasma lipid-lowering drugs. LSRO concluded that "future research will be needed to define the amount and duration of w-3 fatty acid supplementation required to produce the beneficial effects."

FDA agrees that the Burr paper provides valuable evidence consistent with the hypothesized relationship between omega-3 fatty acids and CHD. However, FDA noted in its proposal (56 FR 60663 at 60668) that there are two specific shortcomings in this paper: the absence of separate data for subjects who consumed fish and those who consumed fish oil capsules, and the absence of dose-response data. These data would have provided evidence for specific effect of omega-3 fatty acids. Ideally, other data regarding the subjects diet would also show that there was no difference in consumption of other dietary factors related to CHD. The study design specifically included two such dietary factors, dietary fat and dietary fiber, and the lack of significant effects of these components argues against dietary factors other than omega-3 fatty acids as responsible for the association.

FDA does not consider the Burr paper to have established a beneficial effect of omega-3 fatty acids, although its results are consistent with such an action. The LSRO conclusion indicates that neither the amount of omega-3 fatty acids necessary for beneficial effects nor the duration of their intake has been established. The specificity of the substance responsible for the beneficial effects, the quantitative amount needed to produce the effect and the duration of intake needed to produce the effect need to be established before FDA can authorize a claim linking omega-3 fatty acids to reduction of risk of CHD.

19. Some comments stated that the amount of fish in the Zutphen and Burr studies was so low that the association between fish consumption and reduced CHD mortality could not be explained

by the displacement by fish of other atherogenic foods from the diet.

FDA is not persuaded by these comments. The limitation in these studies is that they did not control for dietary factors associated with CHD, not that fish consumption displaced other atherogenic foods. FDA noted in its proposal that the Zutphen study found significant correlations between fish consumption and other dietary factors (i.e., alcohol, polyunsaturated fats) related to CHD. Comparable correlations were not addressed in the Burr paper because dietary intake data were not reported. Also, the design of the Burr paper was to encourage consumption of fish, which would likely have resulted in a reduction in the consumption of red meat (and, therefore, saturated fat).

20. Two comments discounted the Curb et al. study (Ref. 25), which showed no association between fish consumption and CHD mortality among subjects in Hawaii. The comments stated that the dietary source of fish was likely tropical fish, and since tropical fish feed on coral they have a high content of AA, which would counteract the effect of omega-3 fatty acids.

FDA disagrees with the comments. No data regarding the AA content of the diet in this study, or in other correlational studies, have been reported. Indeed, most epidemiologic correlation studies have not quantified the intakes of omega-3 fatty acids, a fundamental measurement to establish an association between omega-3 fatty acids and CHD. Finally, there is an abstract reporting that the omega-3 fatty acid to omega-6 fatty acid ratio of tropical fish is comparable to or greater than that of fish in higher latitudes (Ref. 237). Thus, the comments' explanation for a negative finding must be considered theoretical.

21. One comment argued that the lack of an association between fish consumption and CHD in two populations in Canada, a prairie province and a coastal province (Ref. 74), was because the prairie population consumed more alcohol and the coastal population smoked more. This comment criticized FDA for not pointing out the cautions raised by the authors about potential confounders like the difference in alcohol consumption.

FDA believes it presented the results of this paper fairly. While the authors reported small differences in smoking (more in the coastal population) and alcohol consumption (more in the prairie population), they stated, "It seems unlikely that these differences are sufficiently large to offset any strong effect of fish consumption." FDA is keenly aware that dietary and

behavioral factors (e.g., smoking, alcohol) must be controlled before meaningful conclusions may be drawn about the effects of omega-3 fatty acids. FDA notes that alcohol consumption was also a confounding factor in a study that reported an association between fish consumption and CHD (Ref. 87).

22. A few comments stated that many of the reported effects come from studies on fish consumption, but that all measured biochemical changes related to CHD that are produced by fish have also been produced with fish oil concentrates.

FDA agrees in part with this comment. The fact that the same biochemical results have been obtained using fish oils rather than fish provides strong evidence that particular biochemical markers are affected specifically by omega-3 fatty acids. Also, since most studies have used fish oils, these results add consistency to the effects reported for studies that used fish. However, FDA disagrees that the comparable findings in studies that used fish oils and fish are sufficient to support the health claim that omega-3 fatty acids reduce the risk of CHD, because the particular biochemical markers affected by both fish and fish oils are not recognized with significant scientific agreement as useful surrogate risk factors for CHD in the general population.

23. One comment argued that the fact that Greenland Eskimos ate diets with half the saturated fat and more polyunsaturated fat than Danes and had much less CHD than Danes strengthens the case for fish oil-derived omega-3 fatty acids.

FDA agrees with the comment that diets lower in saturated fat are consistent with reduced CHD mortality (see the final rule on "Dietary Lipids and Coronary Vascular Disease" published elsewhere in this issue of the **Federal Register**). The differences in saturated fat intake, however, do not strengthen the case for omega-3 fatty acids, because they do not distinguish omega-3 fatty acids from polyunsaturated fats. Rather, the differences in dietary fat intakes strengthens the argument that saturated fat is associated with CHD mortality. The numerous dietary differences between the Greenland Eskimos and Danes make it difficult to ascribe to any single dietary factor the differences in CHD.

24. One comment pointed out that, of the ten prospective studies cited in the proposal (including three in Table 1 of the proposal), six support an inverse relationship between fish consumption and CHD. The comment noted that one

study only showed a relationship in men under 45, and argued that this result is promising because one might expect to find positive effects of long-term fish consumption on CHD in younger, relatively healthy men rather than in older men.

FDA agrees that some but not all reports find an inverse relationship between fish consumption and CHD. FDA does not agree, however that this effect would more likely be noticed among younger, relatively healthy men. Older men would have had a greater duration of intake of omega-3 fatty acids and a greater incidence of CHD than younger men. Both these factors would favor finding an effect in older men rather than in younger men.

25. One comment noted that only two studies found a positive dose-response. Another comment stated that the studies that show no effect are those where the base group already consumed fish, whereas in studies that showed an effect, the base group did not consume fish. A third comment stated that there were data that described an inverse dose-response relationship between serum EPA and CHD deaths among Japanese, but did not identify a particular study.

FDA disagrees that only two studies found a dose response correlation. Each study that response a relationship between fish consumption (or, in one study, the calculated intake of omega-3 fatty acids) and CHD found a dose-response relationship. FDA agrees that most of the dose-response relationships reported suggest that the primary difference in rate of CHD is between those who consume no fish at all and those who consume a small amount of fish, and that there appears to be little additional benefit from consumption of large amounts of fish (Ref. 88). An alternate way of describing these data is that these who consume no fish have an increased risk of CHD. These data merit followup, because a showing that the relationship is due to the omega-3 fatty acids may provide evidence that the long-chain omega-3 fatty acids are essential in the diet.

In its proposal, FDA reviewed a study that described an inverse dose-response relationship between serum EPA and CHD mortality among two groups of Japanese (Ref. 70). FDA concluded that these cross-sectional, correlational data were useful in generating a hypothesis. Other notable dietary factors (including a difference in salt intake of 50 percent) and risk factors for CHD (prevalence of hypertension) also differed between the two groups, so it is not possible to conclude that differences in CHD

mortality were due to differences in dietary omega-3 fatty acids.

26. Two comments stated that FDA had erred in stating that no biochemical data were reported in the Burr paper (Ref. 16).

FDA agrees with this comment, and stands corrected. Bun et al. (Ref. 16) did report that the geometric mean percentages of EPA were 0.59 percent and 0.40 percent in men given advice to consume more fish and those not so advised, respectively, a highly significant difference ($p < 0.01$). The fact that a geometric mean rather than an arithmetic mean was reported implies that there was substantial skewing of the data.

It is not clear from the article whether these differences were for the 6-month time into the trial, or for the end of the trial. The authors did not correlate plasma EPA concentrations directly with myocardial infarction (MI) or CHD deaths.

27. One comment argued that it was highly misleading to state in Table 1 that Kromhout et al. (Ref. 67) reported that, "lean fish, low in omega-3 fatty acids, had some protective effect against CHD," because Kromhout did not distinguish between the effects of lean and fatty fish.

FDA disagrees with this comment. The authors made two statements about lean fish that imply that additional data analyses were conducted, although (as the comment correctly notes) results of these analyses were not included in the paper. The authors wrote, "Lean fish was also inversely related to mortality from coronary heart disease," and "Thus, the inverse relation between lean fish and coronary heart disease cannot be explained by eicosapentaenoic acid."

FDA interprets these comments as a caution to the reader against assuming that EPA was the active component responsible for the observed reduction in CHD among fish-consuming subjects.

28. LSRO included in its report two studies that correlated plasma omega-3 fatty acids with dietary intake of these fatty acids (Refs. 213 and 225). Two other papers reviewed by LSRO but not included in the FDA proposal were correlation studies of mortality from different diseases among Greenlanders and Danes (Ref. 170) and diet-disease correlations in Japan (Ref. 284).

FDA agrees with LSRO's descriptions of these studies. FDA notes that the authors of the studies that correlated intake and plasma levels of omega-3 fatty acids did not relate their data to CHD. The correlation studies of Mortality did not provide any specific data regarding omega-3 fatty acids.

29. One comment provided new dose-response data from additional analyses of data of the Dart study, previously reported in part by Burr et al. (Ref. 16), that related the dietary intake of EPA at 6 months into the trial to the risk of CHD events (heart attacks, or MI's) or CHD mortality. The 947 subjects for whom dietary data were obtained were grouped according to EPA intake; 114 consumed less than 1 g per week (1 g/week), 373 consumed 1 to 2 g/week, and 460 consumed 2 or more g/week. The percentage of subjects that experienced either a nonlethal heart attack or died from a heart attack decreased as dietary EPA increased. For heart attacks the rates were 7.9 percent, 7.0 percent and 6.7 percent, for the less than 1 g/week, 1 to 2 g/week and 2 or more g/week groups, respectively. The percentages in each group who died were 6.1 percent, 5.1 percent, and 4.1 percent, respectively. There were no statistical analyses of these data reported.

FDA notes some limitations in these data as reported that caution against strong conclusions. Most notably, the analysis excluded the events and deaths during the first 6 months of the trial, when about half of all events and deaths occurred. This clearly diminishes the sensitivity of the analysis, and may result in an underestimation of the true effect, since the difference in survival between the group advised to eat more fish and the group not advised to eat more fish was most pronounced, during the first 6 months. Alternatively, if the healthiest subjects were also the most compliant subjects, the reduced death rate in the highest EPA-consumption subjects may reflect the underlying health of those subjects, and the importance of dietary EPA may be overestimated.

Also, the unequal group sizes for this analysis places a greater weight on each subject in the smallest group (less than 1 g/week) than in the other groups. This may be particularly important because the smallest group includes those who consume no fish, and who may differ from fish consumers in other dietary or behavioral factors associated with CHD risk. The sensitivity of the results to small changes in outcomes is shown by example: one fewer death (6/114 rather than the reported number, 7/114) makes the CHD death rate of the less than 1 g/week group equal to the rate in the 1 to 2 g/week group.

Finally, although the dietary intake data at 6 months are useful, this study also assayed plasma fatty acids. Use of plasma EPA (or EPA plus DHA) in the dose-response analysis would have been a more powerful analysis, because it eliminates errors in the diet record data

corrects for losses during food preparation and individual differences in bioavailability of the fatty acids, and integrates intake of omega-3 fatty acids over a longer period than the diet record data.

Therefore, FDA finds these dose-response data to be consistent with the hypothesis that omega-3 fatty acids reduce the risk of CHD, but the shortcomings discussed above limit their usefulness in establishing a relationship between omega-3 fatty acids and risk of CHD.

2. Evidence relating omega-3 fatty acids to intermediate or surrogate markers of CHD

In the proposed rule (56 FR 60663 at 60668), FDA stated that most information about the effects of omega-3 fatty acids on CHD has been derived from clinical trials using concentrated fish oils enriched in EPA and DHA, and in some cases purified methyl or ethyl esters of EPA and DHA. FDA concluded that:

*** there are a few established effects of omega-3 fatty acids from fish oils on thrombosis and hemostasis. Standardized bleeding times are increased, and platelet aggregation and function are reduced. However, direct relationships between the changes in bleeding times or platelet function and risk of CHD have not been established. While there is an established relationship between blood pressure and CHD, it has not been shown that omega-3 fatty acids specifically affect blood pressure in normal subjects in a way that would provide a protective benefit toward the risk of CHD. Effects of omega-3 fatty acids on other markers linked with CHD, e.g., fibrinogen or lipoprotein (a) have not been established. (56 FR 60663 at 60671).

a. Atherosclerosis

i. Blood lipids

30. Numerous comments criticized FDA's focus on blood cholesterol as a surrogate marker for risk of CHD, although one comment noted that such an emphasis would be expected, given the importance of cholesterol in CHD. Another stated that the focus on cholesterol ignores other factors that determine blood cholesterol such as heredity, exercise, and stress, etc.

FDA disagrees with the comments that it overemphasized the importance of studies in which cholesterol was measured as a surrogate marker for CHD. The considerable attention given to blood cholesterol measurements (and measurements of other blood lipids and the protein components of blood lipoproteins) was the consequence of two factors: (1) There was a large number of studies on the effects of fish

oils or fish containing omega-3 fatty acids on these blood lipid measures; and (2) there is general agreement that certain blood lipids are strongly associated with the risk of CHD.

FDA undertook to evaluate findings in these studies whether or not fish or fish oils were used as the source of omega-3 fatty acids and whether or not the outcome measures were generally recognized as predictive of CHD. This approach allowed the identification of biologic activities of omega-3 fatty acids that may be related at some point to risk of CHD, and identified areas where additional research is needed. FDA included in the summary of its proposed rule (56 FR 60663) only measures of generally recognized risk factors. FDA did not intend to imply that data on alternate markers were not considered in its decision.

FDA agrees that other factors contribute to blood lipid measures, but believes that randomization should control for these factors. In nonrandomized studies, these sources of potential bias limit the conclusions that can be inferred from the data. This is an important reason that data from correlation studies do not conclusively establish a relationship between omega-3 fatty acids and risk of CHD.

31. The LSRO report paid considerable attention to changes in blood lipids after increased consumption of omega-3 fatty acids, and reached some conclusions about effects of omega-3 fatty acids on blood lipids that differed from those reached by FDA. LSRO abstracted three studies from before the period covered by FDA review (Refs. 284a, 267, and 257). These studies were considered, in Federal Government and other comprehensive reports reviewed in the proposed, rule and not discussed further by FDA. LSRO also included three studies not reviewed by FDA in its proposal (Refs. 168, 226, and 301). Agren et al. (Ref. 168) studied healthy students randomly assigned to their normal diet (one fish meal per 2 weeks), a fish diet, or a fish diet low in saturated fat for 15 weeks. There was no change in total serum cholesterol on the control diet or fish diet, but the low-fat fish diet produced a reduction in total cholesterol. Jensen et al. (Ref. 226) studied 18 healthy subjects supplemented sequentially for 4-week periods with 4-week washouts between, with fish oils containing 1, 3, and 6 g EPA plus DHA, and found no change in total or LDL cholesterol. Wolmarans studied healthy subjects fed a meat diet or fish diet containing 6.1 g EPA plus DHA for 6 weeks each in a crossover design, and found reduced total cholesterol and LDL cholesterol

during the fish diet phase. There was no difference in total fat of the two diets. However, there was significantly less saturated fat in the fish diet, so it is not certain that the omega-3 fatty acids were responsible for the decrease in the cholesterol measures.

FDA is concerned that the studies abstracted by LSRO do not accurately represent the totality of publicly available scientific evidence. For example, in its proposal, FDA included five studies among normal subjects (Refs. 2, 6, 24, 73, and 143) and three studies among subjects with preexisting lipid or lipoprotein abnormalities (Refs. 18, 73, and 93) not included in the LSRO report that had data for effects of omega-3 fatty acids on plasma lipids or lipoproteins. FDA determined that seven studies that reported changes in total cholesterol had the most rigorous designs and the largest numbers of normal subjects. None of these seven studies (Refs. 6, 9, 14, 49, 54, 73, and 166) in normal subjects found a significant change in total cholesterol after fish oil supplementation. FDA found similar results with regard to hyperlipidemic subjects.

Only two of these seven strongest studies in normal subjects were abstracted in the LSRO text, and two others were not cited at all by this report. LSRO did not distinguish between normal and hyperlipidemic subjects in its summary or conclusions. LSRO summarized the evidence on total cholesterol by stating, "Decreases in total cholesterol *** have also been reported," (emphasis added), without mentioning that the predominant finding is that there is no effect on total cholesterol.

Similarly, FDA stated that the strongest studies among normal subjects (Refs. 6, 9, 14, 49, 54, 73, and 166) found no change in LDL cholesterol, and one reported an increase in LDL cholesterol (Ref. 54). Indeed, most studies on hypertriglyceridemic or hypercholesterolemic subjects reported an increase in LDL cholesterol, following fish oil supplementation (56 FR 60663 et. 60669). Consequently, FDA disagrees strongly with the summary statements in the LSRO report:

Effects of fish oil upon LDL have been variable, in part because of different doses. In normolipidemic individuals, LDL has generally declined significantly. In some patients with primary hypercholesterolemia, consumption of fish (sic) has not resulted in altered plasma cholesterol levels; other studies have shown decreased cholesterol and LDL levels. (Emphasis added.)

32. Two comments stated that FDA had not considered all relevant data on HDL₂ cholesterol, and cited additional

studies that reported increased HDL₂ cholesterol after fish oil

—supplementation. One comment stated that overall HDL cholesterol tends to rise, and cited a review paper by Harm (Ref 62). The LSRO report also concluded that HDL was increased by fish oil supplementation.

FDA disagrees with the comment regarding the overall HDL cholesterol change after fish oil supplementation. The agency considered HDL changes separately for normal, healthy subjects and for hyperlipidemic subjects (56 FR 60663 at 60669). Nearly all studies on normal subjects found no significant change in HDL cholesterol level. Some investigators reported increased HDL₂, but the data on HDL₂ were equivocal.

FDA also disagrees with the conclusions of the LSRO report regarding HDL cholesterol, because it does not represent the totality of publicly available scientific evidence. The LSRO summary states, "In some studies HDL concentrations have actually increased with consumption of fish oil" (emphasis added), not acknowledging that the balance of available scientific evidence on HDL indicates no change. In the review by Harris cited in the comment, the changes in HDL cholesterol in each study were weighted according to the number of subjects in the study, giving per-subject change. This method of pooling data from different studies does not account for the variation of the response of subjects in each study, the amount of omega-3 fatty acids fed, the duration of feeding, or the source of the omega-3 fatty acids. Therefore, it must be considered only an estimate of the effects of omega-3 fatty acids on HDL cholesterol. Harris calculated the average HDL cholesterol change for normal subjects to be an increase of approximately 3 percent, a net change smaller than the usual variability in the test used to measure HDL.

The agency agrees with the comment that not all HDLs data were considered in the proposed rule, although FDA noted (56 FR 60663 at 60669) that some studies among normal subjects found increases in the HDLs fraction of HDL cholesterol, and that these reports were the most promising changes in blood lipids. Of the six references cited by the comments as not included among studies showing increased HDL cholesterol after omega-3 fatty acids, two were published after the time period covered in the proposed rule (Refs. 235 and 252). One other paper not cited by FDA in its proposal, although was published during 1988 (Ref. 291), dealt with insulin-dependent diabetics. The other three papers were cited by

FDA in other contexts, but data from these papers regarding HDL₂ cholesterol levels were not discussed (Refs. 1, 32, and 148).

FDA reexamined those papers that it cited but from which it did not present data regarding HDL₂, together with the newer papers. When fractions of HDL cholesterol have been reported, an increase has generally been found in the HDL₂ fraction (Refs. 1, 32, 148, 235, 251, and 291), with a comparable decrease in the HDL₃ fraction (Refs. 1, 235, and 251). This represents a shift within the HDL fractions toward a lipid-rich lipoprotein, and away from a protein-rich lipoprotein, similar to that reported for LDL, below. This shift has been reported when there is (Refs. 32, 148, 235, and 291) or is not (Refs. 1 and 251) a change in total HDL cholesterol. This raises the possibility that a shift occurred in other studies where total HDL was reported as not changed.

However, the importance of the shift in subfractions of HDL is not clear. FDA noted in its proposal (56 FR 60663 at 60869) that there is evidence that the HDLs fraction is the one most closely linked to risk of CHD. However, the agency was unable to find evidence that there was significant scientific agreement that HDL₂ was the fraction of HDL most closely associated with CHD. The National Institutes of Health's National Heart, Lung, and Blood Institute (NHLBI) consensus development conference on Triglyceride, High Density Lipoprotein and Coronary Heart Disease (Ref. 255), anticipated in the proposal (56 FR 60663 at 60664), concluded that, "It is not known to what extent these alterations of HDL contribute to atherogenesis."

Therefore, data on changes in HDL subfractions after increased consumption of omega-3 fatty acids do not provide a sufficient basis for a health claim, because there is not significant scientific agreement that HDL₂ is directly related to risk of CHD. If the risk of CHD becomes linked with HDL₂, these findings in normal subjects may be of great importance.

33. Many comments indicated that high triglycerides are causally related to decreased HDL, that triglycerides are an independent risk factor for CHD, or that statistical manipulations of data and imprecise measurements of triglycerides obscure the importance of triglycerides as a risk factor for CHD. One comment provided additional citations regarding the relationship between triglycerides and HDL, but these did not bear on risk of CHD. One comment stated that it was generally agreed that triglycerides were not independently associated with CHD.

FDA disagrees with all but the last comment. FDA is aware that there has been, and still is, substantial interest in the potential role of triglycerides in the etiology of CHD (e.g., Ref. 208). Because of the continued interest, the relationship between triglycerides and CHD was the topic of a consensus development conference sponsored by NHLBI on February 26 through 28, 1992. NHLBI had previously addressed this topic in 1983 and concluded at that time that the relationship was controversial. The recent conference (Ref. 255) concluded, "For triglyceride, the data are mixed; although strong associations are found in some studies, the evidence on a causal relation is still incomplete."

FDA agrees that the statistical methods previously used to study the relationship between triglycerides and CHD have lessened the likelihood that triglycerides would be found to be a significant, independent predictor of CHD. Furthermore, the agency believes that study design and analytic measurement methods have contributed to variation in triglycerides that may have resulted in reducing the statistical association between triglycerides and CHD. FDA believes that these sources of variation in triglycerides can be reduced by careful study design and standardized analytical measurement techniques, and also that clinical studies designed to lower triglycerides could provide a basis upon which to reconsider the importance of triglycerides in CHD.

34. Some comments stated that some very recent evidence from the Helsinki Heart Study supports a protective effect of lowering triglycerides, at least for a selected subpopulation of people with a high ratio of LDL cholesterol/HDL cholesterol and very high triglycerides.

FDA agrees that fish oils reduce plasma triglycerides. In its proposal FDA wrote, "The predominant blood lipid effects of fish oils * * * are decreased plasma triglycerides and VLDL." (56 FR 60663 at 60669.) In this regard FDA and LSRO were in agreement. The LSRO summary states, "The most striking effect is lowering of plasma triglyceride and VLDL concentrations."

FDA disagrees, however, that triglycerides have been established as an independent risk factor for CHD. The recent results from the Helsinki Heart Study (Ref. 242) were discussed at length at the NHLBI consensus development conference (Ref. 255). While the reduction in CHD mortality following drug intervention was dramatic (i.e., approximately 7-fold) for a particular subgroup with both elevated

triglycerides and elevated LDL to HDL ratio, this result was obtained by a post hoc analysis of earlier results. Because the combination of factors used to denote the high-risk group (i.e., high LDL cholesterol to HDL cholesterol ratio and high triglycerides) was determined after the data were collected, those results are not the results of the testing of a hypothesis, but are the origins of a new hypothesis. The authors indicate that the cut-off points for the ratio of LDL to HDL and triglycerides chosen were to some extent arbitrary. The actual number of cardiac events in the study was small (e.g., 18 events among 138 subjects in the highest risk subgroup), and the reduction in all-cause mortality due to the lipid-lowering drug, gemfibrozil, was not significant. Finally, independent of LDL to HDL ratio, increased triglycerides alone were not associated with an increased risk of heart attack.

The dramatic reduction of triglycerides by omega-3 fatty acids has resulted in their use in the treatment of a rare genetic hypertriglyceridemia (type V) to prevent noncardiovascular effects of high triglycerides (i.e., pancreatitis), but the usefulness of lowering triglycerides as a general strategy in prevention of CHD is not generally agreed. Therefore, FDA believes that the triglyceride-lowering effect of fish oils on some at-risk persons does not provide a basis for a health claim at this time.

35. Numerous comments indicated that postprandial triglyceridemia is a mechanism of action in the development of atherosclerosis. Some comments indicated that the relationship of elevated triglycerides to risk of CHD would be discussed at the NHLBI consensus development conference (Ref. 255). Others pointed out that LSRO had concluded that elevated very low density lipoproteins (VLDL) and triglycerides were atherogenic. LSRO stated that the reduction of postprandial hyperlipidemia is a "most important anti-atherogenic action." LSRO wrote in the summary that, "Since postprandial lipemia has been identified as an atherogenic risk factor, its prevention by w-3 fatty acids would be a most desirable effect" (emphasis added), and in its conclusions LSRO wrote:

Fish oil has a generally accepted hypolipidemic effect without depressing HDL. This applies most to VLDL and triglyceride, lipids now believed to be atherogenic. There is little doubt that there is reduction of postprandial hyperlipidemia following the ingestion of dietary fat if the background diet contains relatively small

quantities of w-3 fatty acids. This may be a most important anti-atherogenic action.

FDA agrees that fish oils do not generally lower HDL. FDA also agrees that major blood lipid effects of omega-3 fatty acids are reductions of triglyceride and VLDL. The role of omega-3 fatty acids in the reduction of postprandial triglycerides was described in three papers abstracted by LSRO (Refs. 15, 59, and 163). While the first two papers used high levels of omega-3 fatty acids (30 and 9 g of EPA plus DHA/day, respectively), the recent paper used only 5 g of fish oil, containing 1.7 g EPA plus DHA. These studies showed that the concentration of plasma chylomicrons after a high-fat test meal was significantly less if the subjects had been consuming a fish oil diet than if they had been consuming a saturated fat or olive oil supplemented diet. Thus, FDA agrees that fish oils reduce postprandial lipemia.

However, FDA disagrees that there is significant scientific agreement that VLDL and triglycerides are atherogenic, or that the reduction in postprandial hyperlipemia is a most important anti-atherogenic action. Neither the Federal Government nor other authoritative reports have included these blood lipid measures among those they consider to be independent risk factors associated with CHD (Refs. 34 through 36, and 115). Furthermore, postprandial lipemia was discussed at the February 1992 NHLBI consensus development conference. The summary of that conference stated, "Postprandial triglyceride may be more important than the fasting triglyceride levels [to CHD], but little is known about this at the present time." (Ref. 255).

FDA notes that the only paper in the LSRO report cited in support of this hypothesized mechanism of action of omega-3 fatty acids in the prevention of CHD was a review paper published in 1979 (Ref. 305). Therefore, FDA believes that there is not significant scientific agreement at this time that postprandial triglycerides are related to the risk of CHD.

ii. Vessel wall effects

36. One comment indicated that two new studies support the use of omega-3 fatty acids to prevent restenosis, the closing of a mechanically opened blood vessel (Refs. 172 and 259). This comment suggested that FDA discounted the findings of the Dehmer study (Ref. 30) on the basis that it employed simultaneous treatment with drugs and fish oils.

FDA considered the use of omega-3 fatty acids to prevent restenosis to be a drug usage (56 FR 60663 at 60670), and

notes that patients in these studies are under a physician's care. FDA's description of the Dehmer study points out a limitation of the data that is common in other reports of no effect of omega-3 fatty acids in restenosis (Refs. 56, 106, and 121), that the studies have not controlled for generalized effects of PUFA's that are not specific to omega-3 fatty acids. A better balanced experimental design would be comparison of drugs plus omega-3 fatty acids to drugs plus alternate PUFA's (e.g., corn oil).

FDA agrees that the new studies provide some support for the role of omega-3 fatty acids in prevention of restenosis, although neither was designed to distinguish effects of omega-3 fatty acids from effects of omega-6 PUFA's.

Nye et al. (Ref. 259) studied 79 men and 29 women who were referred for angina and underwent coronary percutaneous transluminal angioplasty (PCTA), i.e., a mechanical opening of a closed heart blood vessel. The subjects were randomly assigned to one of three treatments: (1) A combination of aspirin plus dipyridamole (an anti-platelet combination of drugs), (2) olive oil placebo, or (3) 12 milliliters (mL) fish oil containing 3.2 g EPA plus DHA/day. Subjects were restudied 1 year later or before if symptoms recurred, and 93 percent of all subjects were followed for the year. Although there was no significant difference in angina among the groups, the rate of restenosis, defined in this study as a loss of 50 percent or more of the luminal diameter increased by PCTA, was significantly less in the fish oil group (11 percent) than in the placebo group (33 percent).

The use of olive oil as the placebo did not control for effects due to PUFA's (omega-6). Also, it is notable that the restenosis rate in the aspirin group was somewhat higher (17 percent) than in the fish oil group, because aspirin is a much more potent inhibitor of platelet function than EPA in fish oil.

Nonetheless, these results are consistent with an effect of omega-3 fatty acids in reducing restenosis.

The full "Quebec study" was published after the receipt of the comment, but because it was cited in the comment it will be discussed here. In this study, Bairati et al. (Ref. 172) conducted a double-blind, randomized intervention with either fish oil containing 4.5 g EPA plus DHA/day, or olive oil placebo in 205 patients undergoing first PCTA. The treatments were started 3 weeks before the procedure, and continued for 6 months after. Restenosis was assessed angiographically, using a quantitative

computer analysis program. Restenosis was reduced in the fish oil group compared to the olive oil group according to 3 of 4 definitions of restenosis. It was not reduced according to the clinical definition used by Nye et al. (Ref. 259), above, of a loss of 50 percent or more of the luminal diameter increased by PCTA.

This study also collected dietary data. The third of the subjects with the highest consumption of omega-3 fatty acids (0.15 g/day) and the third of the subjects with intermediate consumption of omega-3 fatty acids (0.033 to 0.15 g/day) had significantly lower rates of restenosis than the third consuming the least amount of omega-3 fatty acids. In fact, dietary omega-3 fatty acids (other than the supplement) were associated with a greater reduction in chance of restenosis than was the supplement. This result was somewhat surprising, since the supplement contained 30 times the amount of omega-3 fatty acids in the diet. No differences in rate of restenosis were found according to intake of total fat, polyunsaturated fat, monounsaturated fat, saturated fat, cholesterol, or total seafood consumption. These results suggest that chronic consumption of low amounts of omega-3 fatty acids may be as useful in preventing restenosis as much larger amounts consumed for a few weeks prior to and after PCTA.

In general, the results of Bairati et al. (Ref. 172) and Nye et al. (Ref. 259) are consistent, even though they obtained different results accenting to one identical definition of restenosis. The Bairati et al. study, like Nye et al. 1990, used olive oil as the control. If the mechanism of action of omega-3 fatty acids in restenosis is through competition with AA, this control is suitable, and an omega-6 fatty acid oil would have made the difference due to omega-3 fatty acids even more pronounced. If, however, the mechanism of action is through nonspecific effects of highly unsaturated fatty acids, then a control of a PUFA (e.g., corn oil) might have reduced the apparent effect of omega-3 fatty acids. It is notable that the only study of restenosis that has used a polyunsaturated fat control (an olive oil-corn oil mix) did not find an effect (Ref. 56).

37. Five studies in humans relevant to the action of omega-3 fatty acids on the vessel wall were referenced in comments (Refs. 200, 213, 259, 268, and 277), including two published since the time period covered by FDA's review in proposed rule (Refs. 200 and 268). Hamakazi et al. (Ref. 213) found a slower aortic pulse wave velocity (an

electro-physiologic measurement) in persons from a Japanese fishing village compared to those from a farming village. Other data showed the populations differed in their intake of omega-3 fatty acids. Rapp et al. (Ref. 268) measured the amount of omega-3 fatty acids in the atherosclerotic lesion after consumption of omega-3 fatty acids at a high level (6 percent of calories, 16 to 21 g EPA plus DHA/day) for 6 to 120 days prior to planned surgical intervention, and found that the amount of omega-3 fatty acids in the lesion continued to increase throughout the time of ingestion. Force et al. (Ref. 200) studied the effects of fish oils and aspirin on the production of urinary metabolites of AA and EPA. Fish oil feeding resulted in a slight decrease in the amount of thromboxane A₂ made in the platelet, a decrease in the amount of AA-derived prostacyclin made in the endothelial cell, and an increase in the amount of EPA-derived prostacyclin made in the endothelial cell. Schmidt et al. (Ref. 277) described decreased Bionocyte chemotaxis among hypertensive patients after fish oil feeding. The Nye et al. study is discussed in comment 36 of this document.

FDA considers these studies to be observational, not clearly associating omega-3 fatty acids with risk of CHD. The correlation data of Hamakazi et al. do not indicate a specific role for omega-3 fatty acids. The Rapp et al. data verify that it is possible to incorporate omega-3 fatty acids into preexisting atherosclerotic plaque, but the relevance of incorporated omega-3 fatty acids has not been established. The studies of Force et al. and Schmidt et al. relate to a potential mechanism of action of omega-3 fatty acids, but the importance of these actions in reducing risk of CHD has not been established.

38. Many comments stated that the biochemical and physiological actions of omega-3 fatty acids are anti-atherogenic because they favor vasodilatation and inhibit vasoconstriction. One comment by a manufacturer of omega-3 fatty acids considered these actions have potential for future significance. Two comments cited a list of effects of omega-3 fatty acids, suggesting that each of the effects in the list was anti-atherogenic, and other comments referred to one or more of the components in the list. The listed changes were: decreased thromboxane; increased prostacyclin and leukotriene (LTB₄); decreased fibrinogen, decreased platelet activating factor (PAF);

decreased platelet-derived growth factor (PDGF); decreased superoxide; decreased interleukin-1(TNF); increased endothelium-derived relaxation factor (EDRF); decreased lipoprotein (a) (Lp(a)); reduced inflammatory response; and increased fibrinolytic activity.

The LSRO report stated that other mechanisms, such as cellular growth factors, interleukin-1 and cytokins, and EDRF may be important in the development of atherosclerosis, and be affected by omega-3 fatty acids. However, except for a single in vitro study on PDGF, no data are described in the report regarding these factors, nor is their relevance to human CHD discussed.

FDA addresses fibrinogen, Lp(a), and fibrinolytic activity in comment 46 and in section II.C.2. of this document. FDA does not agree that omega-3 fatty acids produce changes in all of the listed parameters. FDA has determined that for some of these endpoints the changes have not been shown to be specific to omega-3 fatty acids, but may be due to polyunsaturated fats instead. FDA disagrees that the changes brought about by omega-3 fatty acids will prevent atherosclerosis. Most of the data regarding changes in these endpoints brought about by omega-3 fatty acids have been derived from tissue culture or animal experiments, and the relevance to human atherosclerosis has not been demonstrated.

Thromboxanes and prostacyclins are compounds derived from omega-3 fatty acids and omega-6 fatty acids that affect the relaxed state of the blood vessels. Thromboxanes are produced primarily in platelets, and prostacyclins are produced primarily in the endothelial cells of the blood vessels. The thromboxane made from an omega-6 fatty acid called AA, thromboxane A₂ is a potent vasoconstrictor. EPA competes with AA for the enzyme that makes thromboxane A₂, and thereby diminishes the rate of production of thromboxane A₂; the thromboxane made from EPA is a much less potent vasoconstrictor. The prostacyclins made from AA or EPA in the endothelial cells are vasodilators. Thus, the relative amounts of AA and EPA in platelets and endothelial cells play a role in determining the form and amounts of the prostaglandins and thromboxanes that affect the tension of the vessel wall. Excessive constriction may lead to an occlusion, resulting in a heart attack. While there is general recognition that these vasoactive compounds may play a role in the formation of clots and thereby in heart attacks, there is no

agreement about the extent of changes needed in the concentrations of the vasoactive compounds in order to have an effect on heart disease. Changes in the amounts of these vasoactive compounds, produced by consumption of fish oil, are only useful as marker for CHD only insofar as there is significant scientific agreement that the magnitude of the changes is related to CHD. FDA is not aware of any such agreement, nor did the comments provide any evidence of agreement that particular changes in the levels of these vasoactive compounds were related to a reduction in risk of disease. Furthermore, the amount of omega-3 fatty acids needed to produce these changes in humans is not known.

For PDGF the evidence is confined to animal studies (Ref. 201), and the relevance to human disease has only been suggested, not demonstrated. The animal studies on PDGF also did not show that the effect was specific to omega-3 fatty acids. For example, the PDGF effect was observed also after polyunsaturated fats, and was abolished by anti-oxidants, suggesting that any highly unsaturated fatty acids prone to oxidation would have the effect. The experiments on EDRF (Ref. 181) also did not show that the effects were specific to omega-3 fatty acids, since the experiments were earned out in the presence of indomethacin, which, blocks the eicosanoid effects of EPA. In fact, the authors consider changes in membrane fluidity to be a reasonable explanation for the effects. In yet other cases, e.g., TNF, there are conflicting results depending on the species (Refs. 41 and 236), and the findings must be considered preliminary.

FDA considered the effect of omega-3 fatty acids on chemotaxis, one aspect of inflammatory response (56 FR 60663 at 60670). A complete discussion of the role of fish oils in inhibition of the inflammatory process is outside of the scope of this rulemaking, but the relationship between omega-3 fatty acids and inflammatory response could be the subject of a petition for a health claim that includes the necessary information about this relationship.

FDA agrees that the biochemistry of the products formed from the omega-3 fatty acids in vivo (i.e., eicosanoids) have been shown under experimental conditions, usually in vitro, to have pronounced effects on the vessel wall. However, demonstration of isolated biochemical effects is not a sufficient basis upon which to make a claim regarding the outcome of a multifactorial process. Intermediate markers of CHD are useful only insofar as there is significant scientific

agreement that changes in these markers produced by omega-3 fatty acids are causally related to CHD.

b. Thrombosis and hemostasis

39. A few comments stated that the mode of action of omega-3 fatty acids may be through stabilization of arrhythmia, and noted the reduced rate of death after heart attacks (MI's) in the Dart study (Ref. 16). This comment also stated that certain animal data were consistent with this hypothesis. The comments stated that the fibrillation mechanism suggested by DART was compelling, because 60 percent of sudden deaths are caused by ventricular fibrillation following reperfusion. Many commented that data from nonhuman primate models show that omega-3 fatty acids abolish arrhythmias, whereas polyunsaturated fat (safflower oil) had a lesser effect.

FDA disagrees with these comments. FDA's review of the literature regarding the usefulness of omega-3 fatty acids in arrhythmia and ventricular fibrillation found only one study on arrhythmias in humans, and it reported no significant effect of omega-3 fatty acids (Ref. 58). A review in 1989 also concluded that, even among the animal studies, there was no significant difference between omega-3 fatty acids and other polyunsaturated fats on arrhythmias (Ref. 269).

The data from the studies in Nonhuman primates (i.e., the marmoset monkey) were published only as a Nonpeer-reviewed paper in a book (Ref. 188). Two papers by the same author on the same topic were cited in 1990 as in press in a peer-reviewed journal, but have not yet been published. Therefore, FDA regards the data on nonhuman primates as preliminary only. Furthermore, the data for the marmoset monkey were obtained after prolonged feeding for 12 or 24 months with a supplement of DHA-rich fish oil at a level of 8 percent of the diet by weight. FDA calculates that this would provide 2.5 g of omega-3 fatty acids from fish oil/kilogram (kg), over 50 times the usual rate of supplementation in human studies (10 g fish oil or 3 g omega-3 fatty acids/day for a 70 kg subject), and over 300 times the amount of omega-3 fatty acids associated with reduced risk of CHD in the epidemiologic literature (Refs. 16, 38, and 87 report 300 to 660 milligrams (mg)/day). Thus, the relevance of these studies to omega-3 fatty acids in the human diet is questionable.

FDA is aware of in vitro data that show a specific protective effect of EPA against toxicity of heart muscle cells in culture. These results provide a

biochemical basis for the hypothesized stabilization of cardiac arrhythmias by omega-3 fatty acids. Although, this study (Ref. 212) was performed, in vitro on heart cells from rats, it showed that the protective effect was specific to omega-3 fatty acids (EPA) because a similar effect was not obtained when a highly unsaturated omega-6 fatty acid (AA) was used instead.

FDA also regards the evidence the Burr study of reduced death following a heart attack among men, advised to increase fish consumption as consistent with a stabilization of arrhythmias (Ref. 16). FDA agrees that this postulated mechanism of action is of great potential public health significance. However, the agency finds the clinical data available at this time are not in agreement with animal and in vitro data. Because the clinical data are not in agreement with these other types of data and because of the limitations in the animal studies, FDA concludes that there is not sufficient basis for protective effect specific to omega-3 fatty acids on arrhythmias, and, therefore, CHD in humans.

40. One comment criticized the 6-week clinical study by Hardarson et al. that found no effect of omega-3 fatty acids on arrhythmias (Ref. 58), arguing that the time for incorporation of omega-3 fatty acids into heart phospholipids was too short for an effect to be observed.

FDA agrees in part mid disagrees in part with this comment. Generally, the time needed for incorporation of omega-3 fatty acids into heart phospholipids is short; studies in animals show such incorporation, in a period of weeks (Ref. 249). In the Hardarson study (Ref. 58), a substantial amount of cod liver oil was fed (20 mL/day) and a 230 percent increase in plasma phospholipid EFA was found. There was no trend toward reduced arrhythmias. Other data, however, show that although plasma phospholipids increase the omega-3 fatty acid content during the first few weeks of supplementation, the incorporation of omega-3 fatty acids in human atherosclerotic plaque continues to increase through 120 days (Ref. 268). Therefore, FDA agrees with the comment the supplementation period in the Hardarson study (Ref. 58) may have been too short to find an effect of fish oils on occurrence of arrhythmias. Also, the agency notes that the absence of a difference in CHD mortality during the first 6 weeks of the Burr study (Ref. 16) is consistent with the hypothesis that prolonged intake of omega-3 fatty acids (longer than 6 weeks) is needed to observe an effect on arrhythmias or

other mechanisms that reduce CHD mortality. FDA agrees that effects of long-term consumption of omega-3 fatty acids on arrhythmias, other platelet or vessel wall functions, and even some blood lipid measures have not been sufficiently studied.

i. Bleeding times

41. Two comments stated that there is no evidence of increased bleeding even among patients who had ingested 6 to 8 g of EPA plus DHA/day and underwent emergency surgery, coronary artery bypass surgery or angioplasty. The comments argued that increased bleeding has not a safety concern.

FDA agrees that there are few reports of excessive bleeding after ingestion of omega-3 fatty acids. However, FDA notes that the cited reports are for subjects with CHD, and evidence of the lack of excessive bleeding complications in this population is not sufficient to assure safety of omega-3 fatty acids in the general population. FDA believes that changes in bleeding due to consumption of omega-3 fatty acids remains a valid safety concern (see comment 52 of this document).

ii. Platelet aggregation

In the proposal, FDA stated:

The relationship between platelet aggregation and the risk of heart attacks or CHD death in the general population is an important line of evidence that would support drug claims and perhaps health claims for omega-3 fatty acids. Although there is some evidence that changes in platelet aggregation may help prevent second heart attacks * * * it has not been shown that changes in platelet aggregation in the general population will reduce the risk of CHD. (56 FR 60663 at 60670.)

The agency added: "What has not been established, however is that platelet aggregation is a bona fide surrogate risk factor for CHD in the general population," (56 FR 60663 at 60672.)

42. Many comments argued that platelet aggregation is completely substantiated as a marker for risk of CHD, based on the results of the Physicians' Health Study (Ref. 66). One comment qualified this conclusion stating that the primary effect of omega-3 fatty acids in vivo was to reduce platelet deposition at sites of aortic lesions.

FDA acknowledges that aspirin studies provide evidence that platelet aggregation is a risk factor for CHD. The effect of aspirin in inhibiting platelet function has been shown. Among persons who have already had an MI, aspirin is effective in preventing a second infarction. FDA has proposed that aspirin be used to reduce the risk

of death and/or nonfatal heart attack in patients with previous infarction or unstable angina pectoris as a professional labeling indication (provided to health professionals, but not to the general public), in the tentative final monograph for over-the-counter internal analgesic, antipyretic, and antirheumatic drug products (November 16, 1988, 53 FR 46204 at 46259). However, FDA does not consider the effects of aspirin in the Physicians' Health Study sufficient to establish that dietary omega-3 fatty acids would have the same effect in the general population. The Physicians' Health Study did not evaluate omega-3 fatty acids. The study population was highly selected; the rate of heart attacks was approximately 10-fold lower than in the general population, and cardiovascular mortality was only 15 percent of that expected for the general population of white men of the same age. Also, the results of the Physician's Health Study are not as straightforward as presented in the comments. The chairman of the Physicians' Health Study reported that there was a reduced risk of MI in the aspirin group, predominantly in nonfatal MI, but that there was no significant effect on overall cardiovascular mortality (a 2 percent reduction, not statistically significant) (Ref. 66). In addition, the aspirin group in this study had a greater number of sudden deaths (Ref. 282).

In the other primary prevention trial (Ref. 265), aspirin did not have any significant effect on heart attacks, on stroke, or on total vascular mortality. There was a significant increase in disabling stroke in the group taking aspirin.

On the basis of these studies there has not been an endorsement of the use of aspirin as a prophylactic measure against CHD by the general population by the American Heart Association or by the Canadian Medical Association (Ref. 187). Notably, "1992 Heart and Stroke Facts" published by the American Heart Association (Ref. 169) makes no reference to platelet aggregation as a risk factor for heart attacks (although sticky platelets are mentioned to be a consequence of cigarette smoking in the section on stroke), nor is aspirin discussed as an option for CHD prophylaxis, even though other drug and surgical treatments are discussed.

Therefore, FDA concludes that there is not significant scientific agreement at this time that platelet aggregation is a surrogate marker for CHD in the general population.

43. The LSRO report, submitted as a comment, contained abstracts of 19 studies in humans that contained data

regarding changes in platelet function following omega-3 fatty acid consumption. LSRO concluded that omega-3 fatty acids prevented platelet aggregation.

In its proposal, FDA stated: "Platelet aggregation is generally considered to be decreased by fish oil consumption." (56 FR 60663 at 60670.) The agency also stated: " * * * platelet aggregation and function are reduced: However, direct relationships between the changes in * * * platelet function and risk or CHD have not been established." (56 FR 60633 at 60671.) Thus, FDA agrees with the conclusions of LSRO about effects of omega-3 fatty acids on platelet aggregation.

Two of the studies described by LSRO were not considered by FDA in its review, because they were published before 1988, and had been considered by Federal Government and other authoritative reports. One study (Ref. 227) used a large amount of fish oil (50 mL/day) not reasonably related to normal dietary intake. The other study (Ref. 211) involved 13 insulin-dependent diabetics, and therefore is of questionable relevance for the general population.

In its proposed rule, FDA considered 13 of the other 17 studies that were abstracted by LSRO. One of the four studies not addressed by FDA was a study on the effects of added vitamin E to fish oil on fibrinogen and fibrinolysis (Ref. 210). Two papers (Refs. 234 and 244) were published after the time period covered by FDA review. Marckmann et al. (Ref. 244) compared the effects of a fish diet and a lean meat diet on plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1) and the activity of the inhibitor (PAI-1 activity), Li and Sterner (Ref. 234) described changes in in vitro platelet adhesion after fish oil supplementation. The fourth paper was an uncontrolled observation study that found a high frequency of nosebleeds in adolescents supplemented with fish oils (Ref. 189).

Six other papers on thrombosis were not described in the LSRO text, but were included in the table (Refs. 203, 204, 209, 226, 245, and 254). Of these six, one was not relevant to the nutrient-disease relationship (Ref. 245) because it did not study EPA and DHA. Jensen et al. (Ref. 226) found no significant change in bleeding times in normal subjects after 1, 3, or 6 g EPA plus DHA/day in healthy subjects. Green et al. (Ref. 209) found no change in platelet aggregation or platelet count in 27 hyperlipidemic subjects in a randomized double-blind placebo controlled crossover trial. The treatments were 15 g/day fish oil

containing 4.3 g EPA plus DHA and a 50:50 mix of corn:olive oil, with each treatment lasting 8 weeks, and a 4-week washout between. Blood viscosity was decreased by fish oil, Gazso et al. (Ref. 204) found decreased platelet aggregation in healthy subjects after consumption of EFamol-marine compared to olive oil in a double-blind randomized crossover study. These results of studies confirm others cited by FDA. The other studies (Refs. 203 and 254) pertained to regulators of bleeding and are discussed below.

Eight papers on platelet fraction were reviewed by FDA but not by LSRO (Refs. 2, 6, 18, 24, 73, 93, 131, and 143). Three studies were uncontrolled (Refs. 18, 93, and 143), while two were randomized (Refs. 2 and 131). Three were randomized, double-blind, placebo-controlled trials that used saturated vegetable oil (Ref. 6), vitamin E (Ref. 24) or wheat germ oil (Ref. 73) as the placebos. The two studies that used vegetable oil or vitamin E as controls found a reduction in platelet aggregation after omega-3 fatty acids, where no difference was reported in the trial that used a wheat germ oil placebo, although the data were not provided in this paper.

FDA and LSRO reached the same conclusions with regard to the effects of omega-3 fatty acids on platelet function. LSRO also concluded that platelet survival is also enhanced, but the only two studies published since 1987 that reported increased platelet survival (Refs. 94 and 144) were both uncontrolled, so the effect cannot be attributed specifically to omega-3 fatty acids.

44. One comment agreed in principle with the agency's assessment of the effects of omega-3 fatty acids on bleeding times, platelet aggregation, regulators of bleeding and blood pressure. The comment, pointed out the extent of inhibition of platelet adhesion is as much, as 60 percent, a marked reduction in adhesion.

FDA agrees that the reported extent of reduction of platelet adhesion omega-3 fatty acid intake is remarkable (Ref. 234). The agency notes that this effect appears specific to omega-3 fatty acids at reasonable intake levels. FDA notes that animal studies (Refs. 230 through 233) published since the proposed rule provide evidence of reduced platelet adhesion to blood vessel endothelium in vivo in response to agents that provoke such adhesion. Because of the magnitude of the effect of omega-3 fatty acids on platelet adhesion, FDA considers this action of omega-3 fatty acids on blood platelet function to have great potential with

regard to the development of atherosclerosis and the risk of CHD. However, as for platelet aggregation, FDA does not believe that there currently is significant scientific agreement that platelet adhesion is an accepted risk factor for CHD in the general population.

iii. Regulators of bleeding

In its proposal (56 FR 60663 at 60670 through 60671), FDA reviewed data on the effects of omega-3 fatty acids on other factors that are involved in the regulation of bleeding—fibrinogen, fibrinolytic activity and Lp(a)—and that have been associated with CHD.

45. Some comments, including the LSRO report, stated that omega-3 fatty acids increase fibrinolysis.

FDA disagrees with these comments. FDA found that there was no clear relationship between omega-3 fatty acids and factors involved in dissolving blood clots (56 FR 60663 at 60671). FDA noted that the data did not establish that omega-3 fatty acids reduced fibrinogen, because most studies did not control for other factors that might have reduced fibrinogen, e.g., other PUFA's.

FDA has reviewed the relevant studies again, as well as studies brought to its attention in the LSRO report. LSRO cited three papers on fibrinogen or fibrinolysis not cited by FDA. One placebo (vitamin E) controlled study found no change in fibrinolytic activity (Ref. 210). Mullertz et al. (Ref. 254) supplemented seven healthy adults with 0.55 g ERA plus DBA/day for 21 days and found increased levels of PAI-1, but no change in t-PA, suggesting that fish oil decreased fibrinolytic capability. Cans et al. (Ref. 203) reported no change in fibrinogen concentration after EFamol-marine compared to corn, oil, which is rich in polyunsaturated fat. These studies do not support the conclusion that omega-3 fatty acids reduce fibrinogen, or increase fibrinolysis.

The selection of studies abstracted by LSRO may not have represented the publicly available scientific evidence. For example, five papers abstracted found either a decrease in fibrinogen or an increase in fibrinolytic activity (Refs. 57, 71, 98, 104, and 117). In contrast, two studies found no change in fibrinolytic activity (Refs. 150 and 166), and only one found increased fibrinogen (Ref. 144), leaving the impression that omega-3 fatty acids usually have been reported to enhance fibrinolysis.

However, three other studies not abstracted by LSRO but included in their tables reported no effect of omega-3 fatty acids on fibrinogen compared to corn oil (Refs. 10, 118, and 203). One

found a decrease compared to olive oil (Ref. 49) and one found a decrease compared to soybean oil only when 30 mL of fish oil were consumed, but not when 15 mL were consumed (Ref. 57). Additional well-designed studies not cited by LSRO, but considered in the FDA proposal, reported no change (Ref. 24) and an increase (Ref. 131) in fibrinolytic activity.

Therefore, FDA stands by its earlier conclusion that the publicly available scientific evidence does not support a relationship between omega-3 fatty acids and decreases in fibrinogen or increases in fibrinolysis. This conclusion is supported by findings that consumption of other PUFA's have effects comparable to those produced by consumption of omega-3 fatty acids.

46. Two comments cited unpublished data by Kostner and Herrmann, reporting reduced Lp(a) after fish oil consumption.

FDA was unable to find the full paper by these authors showing the decrease in Lp(a). FDA did find a paper by these researchers published in 1991 (Ref. 241) that reported no effect of fish oils on Lp(a) and did not cite conflicting work from their laboratory.

iv. Blood pressure

In its proposal, FDA considered the relationship between omega-3 fatty acids and blood pressure, one of the recognized risk factors for CHD. FDA stated:

These results for effects of omega-3 fatty acids on blood pressure of normal subjects are ambiguous. Some studies found a reduction in systolic blood pressure after consumption of fish oils containing omega-3 fatty acids, whereas others did not. None of the studies found a significant reduction in diastolic blood pressure. Therefore, it also remains to be established that the normal, healthy population will reduce their risk of CHD via a reduction in blood pressure following consumption of omega-3 fatty acids.

(56 FR 60663 at 60671.)

FDA also stated that it was not known whether or not the magnitude and duration of the effect would persist after longer term supplementation. FDA recognized that studies among hypertensives found an effect more consistently than studies among normal subjects, although sometimes large amounts of fish oils were used.

47. Some comments considered the effects of omega-3 fatty acids on hypertension as evidence, of a reduction in CHD risk. Other comments called for FDA to reassess the studies on blood pressure. One of these comments that the results of studies on blood pressure are not "completely

ambiguous. One comment agreed in principle with the agency's assessment of the blood pressure studies. One comment considered a number of animal models to be relevant for hypertension. The LSRO report also considered the evidence relating omega-3 fatty acids to blood pressure to be important in relation to CHD. The LSRO report concluded that, "Fish oil probably has a mild hypotensive effect, especially in high doses."

FDA disagrees that the publicly available scientific evidence supports a relationship between omega-3 fatty acids and hypertension. At best, as stated in the proposal, the data are ambiguous. Qualifiers are needed to indicate that the reductions in blood pressure have not generally been shown to be specific to omega-3 fatty acids. Also, many valid studies have reported no effect.

LSRO reported a total of 13 studies on hypertension. Four were published before 1988, and were not reviewed by FDA in the proposed rule. Three of these studies used fish as the source of omega-3 fatty acids and therefore did not show the effect specifically to be due to omega-3 fatty acids. In fact, in one study (Ref. 292), the control diet of meat produced a decrease in blood pressure comparable to that of the fish diet. The study that used fish oils (Ref. 271) used an olive oil control, rather than an oil high in PUFA'S. This study is the only study to show an effect of omega-3 fatty acids on diastolic blood pressure in normal subjects.

Of the other 10 studies on hypertension described in the LSRO report, 6 were also reviewed by FDA (Refs. 11, 57, 80, 85, 95, and 101). The LSRO and FDA interpretations of the results from these papers did not differ in any significant regard, except that FDA specifically noted that two of these studies (Refs. 85 and 95) used very high amounts (50 mL) of fish oil to show the effect. In fact, FDA singled out the Bonaa et al. (Ref. 11) and Kestin et al. (Ref. 80) studies as well-designed studies that showed an effect specific to omega-3 fatty acids in hypertensive and normal subjects, respectively (56 FR 60663 at 60671).

The LSRO report reviewed four papers not originally reviewed by FDA (Refs. 190, 285, and 299), including one study on linolenic acid outside of the scope of the definition of omega-3 fatty acid, as used in this regulation (Ref. 262). Two other papers that appeared in the LSRO table but not in the text (Refs. 203 and 247) were also not reviewed by FDA in its proposal.

FDA agrees with the LSRO interpretation of the Wing et al. study

(Ref. 299), where subjects remained on blood pressure lowering medications and no effects of added fish oils were observed.

FDA disagrees with the LSRO descriptions of the Singer and Cobiac studies. The placebo in the Singer study (Ref. 285) was olive oil, but this was not pointed out in the LSRO text. The reduction of blood pressure observed after fish oil, therefore, may have been due to a general unsaturated fatty acid effect not specific to omega-3 fatty acids. In the description of the Cobiac et al. study (Ref. 190), LSRO did not note that fish oil treatment alone (without simultaneous reduction of salt) had no effect on blood pressure.

Two other studies were cited in the LSRO tables but not in the text and were not included in the FDA review. Neither of these found an effect on blood pressure. Gans et al. (Ref. 203) used a randomized double-blind, placebo-controlled design and found a reduction in diastolic blood pressure for both fish oil and corn oil (placebo). Meland et al. (Ref. 247) carried out a randomized, double-blind multicenter trial among 40 mildly hypertensive subjects, using 6.8 g EPA plus DHA/day, but found no difference in blood pressure compared to a 50:50 olive:corn oil control.

Three other large and appropriately controlled studies not in the text of the LSRO report but included in its table were also reviewed by FDA. Two randomized studies on normal subjects (Refs. 9 and 49) and one controlled study among mildly hypertensive subjects (Ref. 20) reported no differences in blood pressure attributable to omega-3 fatty acids.

FDA reviewed in its proposal three other randomized, double-blind, placebo-controlled studies among healthy subjects that were not included in the LSRO review. Two of these studies were on normal, healthy subjects (Refs. 6 and 24) and found a decrease in systolic blood pressure compared to a saturated vegetable oil or vitamin E, respectively. The third study (Ref. 73) found that omega-3 fatty acids did not affect blood pressure in hypertensives or normal men compared to wheat germ oil.

Therefore, FDA concludes that the evidence of an effect of omega-3 fatty acids on blood pressure in normal subjects is ambiguous, because some studies reported a blood pressure lowering effect, whereas other equally well-designed studies found no specific effect. Studies among hypertensives found an effect more consistently than studies among normal subjects, although sometimes large amounts of fish oils were used, and many studies

did not show that omega-3 fatty acids were more effective than other polyunsaturated fats.

48. Comments stated that other lines of evidence were not discussed in the proposal. Examples given were changes in plasma viscosity, increased vascular compliance, and reduced white blood cell (WBC) count.

FDA disagrees with the comment with respect to plasma viscosity and vascular compliance. In its proposed rule, FDA acknowledged that plasma viscosity was decreased and red cell deformability was increased by omega-3 fatty acids, but that the importance of these effects on the risk of CHD had not been established (56 FR 60663 at 60670).

The agency agrees that it did not systematically consider WBC count among the effects produced by omega-3 fatty acids. WBC count was not included among the actions of omega-3 fatty acids considered in major reviews. FDA notes that WBC count has only recently been identified as associated with risk of CHD by the Caerphilly Collaborative Heart Disease Study (Ref. 301a). Only two papers among literature from 1988 to present have reported a reduction of WBC count after fish oil supplementation (Refs. 183 and 253).

3. Other relevant information

a. Animal studies

49. Numerous comments asserted that animal studies did not receive an appropriate amount of discussion. One of these same comments stated that animal studies are not sufficient to support the claim, and that clinical trials on effects of omega-3 fatty acids directly on CHD are needed. One comment criticized FDA's review of animal studies because the negative findings have been in inappropriate models and should not have been discussed. Another comment stated that they did not believe that there is an appropriate animal model for human cardiovascular and CHD. The LSRO report considered animal studies to provide important evidence for an anti-atherogenic effect of fish oils, stating, "Omega-3 fatty acids have been shown to retard the development of the atherosclerotic plaque in experimental animals including the pig and rhesus monkey."

FDA agrees that the evidence from studies in animals warrants additional discussion. FDA has reviewed here those animal studies that were cited in its proposed rule and those that were cited in the LSRO report that were relevant to the development of atherosclerosis. Other animal studies relevant to the development of

atherosclerosis, and animal studies on aspects of CHD other than atherosclerosis are reviewed under section II.C.3.a. or this document.

In its proposal, FDA cited eight animal studies and one abstract on the development of atherosclerosis that were not included in the LSRO review (Refs. 19, 51, 65, 81, 97, 123, 126, and 151); seven of which reported either no beneficial effect or an adverse effect in fish oil supplemented animals. Only one animal study on the effects of omega-3 fatty acids on restenosis was abstracted by LSRO, although the others cited by FDA were described in the LSRO tables.

Three studies in nonhuman primates have been reported (Refs. 27, 47, and 116). In the Davis et al. (Ref. 27) and Parks et al. (Ref. 116) studies, the polyunsaturated fat intake was higher in the fish oil groups, and polyunsaturated fat is known to lower total plasma cholesterol. Also, the control diet of the Davis et al. study had more saturated fat than the fish oil diet. Thus, the effects on atherosclerosis may not have been specific for omega-3 fatty acids.

In these studies, the total cholesterol values for the fish oil groups were substantially lower than for the control group, which may explain the observed differences in atherosclerosis. In support of this interpretation, Parks et al. (Ref. 116) noted that one of monkeys fed the fish oil diet responded differently than the other 11 monkeys fed fish oil. This one monkey had a plasma cholesterol level comparable to that of the lard-fed control monkeys, and also had atherosclerosis comparable to the lard-fed monkeys.

Changes in total cholesterol levels were noted by authors of another study in pigs that showed a reduction in atherosclerosis concomitant with a reduction in time-weighted total cholesterol (Ref. 81). Since cholesterol concentrations are not changed by fish oils in humans, animal studies where fish oil treatment lowered total cholesterol levels are of questionable relevance to the role of omega-3 fatty acids in the development of human atherosclerosis.

A recent study (Ref. 47) in nonhuman primates (vervet) compared the effects of fish oil supplementation to sunflower oil supplementation in either an atherogenic diet (high fat, low polyunsaturated fat to saturated fat ratio, high cholesterol) or, following the atherogenic diet, in a therapeutic diet (low fat, high polyunsaturated fat to saturated fat ratio and low cholesterol). Animals in each diet group were matched for serum cholesterol. Sixteen separate measures of atherosclerosis

were scored, including various measures of the extent of plaque, loss of endothelium, intimal thickening, and inflammation. Overall there was no benefit of fish oil; in some cases, the atherosclerotic measure indicated more disease in the fish-oil fed animals.

The LSRO report considered the amount of fish oil in the diets in this experiment (1.3 to 1.8 percent of calories) too low to observe an effect. In fact, FDA calculates a lower percent of calories from fish oil (1.0 to 1.5 percent) than calculated by LSRO. This level is about half the amount used in short-term human studies (i.e., 10 mL/day), and FDA agrees that the low level makes it less likely that an effect would be observed than if a higher amount had been used. However, diets were supplemented for a prolonged period of time (20 months) and in the therapeutic diet other dietary factors were also changed that might have made the effects of omega-3 fatty acids more noticeable (e.g., ratio of polyunsaturated to saturated fatty acids). Finally, the fact that there were differences between the fish oil-supplemented group and the polyunsaturated fat group while on either the atherogenic or therapeutic regimens suggests that there was sufficient sensitivity in the experimental design to detect protective effects of omega-3 fatty acids.

In some animal studies that showed a protective effect of fish oils, an invasive procedure was used to accelerate atherosclerosis, either mechanical injury (Ref. 122) or vein grafts (Refs. 90 and 186). These studies may be most relevant to the late stages of atherosclerosis, and to CHD in humans following invasive procedures. All of the animal studies cited in the LSRO report except Kim et al. (Ref. 81) and Fincham et al. (Ref. 47) did not control for PUFA's, so the effects observed have not been shown to be specific to omega-3 fatty acids. Additionally, the level of use of fish oils has been high, e.g., 22 percent of calories in Parks et al. (Ref. 116) and 25 percent of the diet in Davis et al. (Ref. 27), which limits the extrapolation of findings in these studies to levels that might be reasonably consumed by humans.

FDA disagrees with the summary of the literature in animals, as expressed in the LSRO-report, because that report fails to mention important limitations in the data. FDA notes that most studies did not have an adequate design to show specificity of effects as due to omega-3 fatty acids. Furthermore, reductions in total cholesterol in the fish oil fed animals may explain the reported reductions in atherosclerosis. Since reasonable amounts of fish oils in

human diets do not alter serum cholesterol concentrations, the results from these animal experiments are of questionable importance regarding human atherosclerosis. Finally, LSRO did not review numerous animal studies that found no effect or an adverse effect of supplementation with fish oils, and therefore the LSRO conclusion does not represent the totality of publicly available scientific information.

With these qualifications in mind, FDA notes that some of the reported effects of the dietary interventions with fish oils on the development of atherosclerosis have been dramatic. Also, FDA recognized that animal studies are of great importance for study of long-term effects on chronic diseases of consumption of amounts of omega-3 fatty acids, particularly in amounts that might be obtained in a reasonable diet. Therefore, FDA encourages further research in this area using rigorous study designs and amounts of omega-3 fatty acids reasonably available in a normal diet to elucidate any effects specific to these fatty acids.

After closer scrutiny of the animal studies cited in FDA's proposal and in the LSRO report, the agency has reached the same conclusion that it reached in its proposed rule: there are some data in studies from animals which suggest the possibility of a beneficial effect of omega-3 fatty acids on CHD; however, the data are equivocal. (56 FR 60663 at 60671.)

b. Safety considerations

50. One comment stated that the increases in LDL cholesterol observed were a chance occurrence, and another stated that increased LDL should not be considered an adverse finding in light of the results of the Burr study.

FDA disagrees with this comment. FDA found that increased LDL cholesterol was ordinarily found when hyperlipidemics or diabetics were given fish oil supplements. This may be due in part to the fact that fairly large amounts of omega-3 fatty acids (i.e., 5 g EPA plus DHA/day or more) were used in these studies. Increased LDL is not ordinarily seen in the studies on normal subjects.

FDA does not consider the Burr study (Ref. 16) to have established that omega-3 fatty acids reduce the risk of CHD, and therefore remains concerned that increases in LDL cholesterol could be adverse for some subjects. FDA notes that concern about increased LDL cholesterol was expressed in the report of the NHLBI consensus development conference (Ref. 255).

51. One comment stated that it was inappropriate to consider adverse effects

in subpopulations without describing the advantages of omega-3 fatty acids in those same populations.

FDA disagrees with this comment. As stated in the final rule on general requirements for health claims, published elsewhere in this issue of the *Federal Register*, it would be a violation of the agency's responsibility under the act to authorize a health claim about a substance without being satisfied that the use of the substance was safe. The agency attempted to examine all available scientific evidence regarding the effects of omega-3 fatty acids. FDA separated out the potential adverse effects discovered during its review, because it wanted to draw attention to these issues as impediments to a health claim for omega-3 fatty acids and CHD. Such potential adverse effects must be resolved, and may be important in setting the conditions under which FDA would allow a health claim to appear on the label and labeling of foods and food supplements.

52. Two comments stated that the safety issues raised in the Mitre Corp. report (Ref. 72) were outdated but did not indicate which issues, or suggest why they were outdated.

FDA recognizes that there has been considerable debate regarding the clinical importance of bleeding times since the publication of the Mitre Corp. report (Ref. 72). However, the agency believes that the issues raised in that report have been restated in subsequent literature, and that all issues of safety are important in deciding whether or not to authorize a health claim.

53. A few comments recommended that FDA balance the benefits of reduced risk of CHD against the risk of reduced glycemic control among diabetics when deciding whether or not to authorize a health claim. One comment stated that physicians could adjust the dose of insulin if omega-3 fatty acids reduced their glycemic control, but another comment stated that glycemic control must be considered a real adverse effect.

FDA agrees that limitations on the use of a substance by a subpopulation (e.g., diabetics) do not necessarily exclude a substance from bearing a health claim for the general population, because the claim may be appropriately restricted. However, FDA agrees that the loss of glycemic control is a potentially serious adverse effect that must be fully addressed before a health claim could be authorized.

54. Another comment stated that a major concern about omega-3 fatty acids not mentioned in the proposed rule is that they may be oxidized and, as

oxidized products, may have adverse effects.

FDA agrees that oxidation of omega-3 fatty acids is a concern. In fact, there are many studies that have been reported since the publication of the proposed rule, or that were not included in FDA's literature review, that indicate such a concern (Refs. 184, 210, 217, 229, 240, 248, 263, 283, and 290).

Antioxidants have been successfully added to supplements and may be adequate to protect the omega-3 fatty acids in foods. It may be necessary to establish conditions that protect against oxidation of omega-3 fatty acids and incorporate those conditions into any future regulation authorizing health claims for omega-3 fatty acids.

55. A related comment indicated that the majority of the fish oil preparations that have been used are severely oxidized, including National Institutes of Health Fish Oil Test Materials.

However, no data regarding the extent of oxidation, the nature of the oxidation products, or the physiologic action of these products was provided.

FDA agrees with this comment. Many of the biologically active products of omega-3 fatty acids are oxidation products. Oxidation of test materials may explain some contradictory findings in the literature.

56. One comment pointed out that increased prothrombin times and possibility of increased stroke were not discussed.

FDA agrees with this comment. FDA did not specifically review data on prothrombin times, although data on bleeding times as a measure of hemostasis were discussed for both normal subjects and for subjects with risk factors for CHD. The importance of the increase in bleeding time brought about by supplemental fish oils or increased fish consumption is not clear. FDA noted in the proposal that most reports suggest that serious bleeding is not an issue in patients supplemented with omega-3 fatty acids, and also that standardized bleeding times do not closely correlate with clinically significant bleeding. However, concerns about untoward bleeding after supplemental fish oils have been raised in the literature (Refs. 106, 120, and 189).

FDA did not discuss the possibility of increased occurrence of stroke as a consequence of increased consumption of omega-3 fatty acids. The papers that reported a correlation between high consumption of omega-3 fatty acids from fish and other marine animals and low rate of CHD mortality also noted an increase rate of stroke, particularly hemorrhagic stroke (Refs. 8 and 84).

Also, the possibility of increased rates of stroke are raised by the data from studies on aspirin (Ref. 66).

Thus, FDA considers these potential adverse reactions to be legitimate concerns, primarily in the context of very high intakes of omega-3 fatty acids.

57. One comment stated that even if adverse effects were only suspected in a medical disorder, pronounced warnings or contradictions would be required.

As noted above, the agency must be satisfied that the use of a substance is safe before it will authorize a health claim about the substance. Thus, suspicions about potential adverse effects would need to be resolved prior to the authorization of a claim. Certain health claims may require appropriate qualifications as a way of minimizing potential safety concerns.

C. New Scientific Data

To determine whether or not new scientific data published since the proposed rule provided a basis for modifying FDA's conclusions regarding the relationship between omega-3 fatty acids and risk of CHD, FDA conducted a search of the scientific literature for relevant studies. Reviews published since the period covered in the literature review in FDA's proposed rule were used to identify recently published studies.

1. Epidemiologic studies

a. Cross-sectional studies and surveys (Table 1)

Bulliyya et al. (Ref. 185) found lower total serum cholesterol and higher HDL cholesterol in a fish-consuming coastal village population than in a nonfish-consuming population from the interior of India. These correlational data are consistent with a beneficial effect of omega-3 fatty acids on blood lipids, but many possible confounding variables prevent strong conclusions regarding a specific role for omega-3 fatty acids.

In a retrospective study, Popeski et al. (Ref. 266) found that women from communities with higher marine oil consumption had significantly lower diastolic pressure in the last 6 hours of pregnancy than women from communities with low fish oil consumption. Pregnancy associated hypertension was 2.6 times more common in communities with low fish consumption. These correlational data are consistent with an effect of omega-3 fatty acids on blood pressure in this particular situation. Again, many possible confounding variables prevent strong conclusions regarding a specific role for omega-3 fatty acids.

b. Prospective studies (other than intervention studies) (Table 1)

Bjerregaard and Dyerberg (Ref. 176) reported age-standardized mortality rates per 10,000 person-years for CHD in men in Greenland settlements (5.3) as half of that reported for men in Denmark (10.0). There was an increasing rate of CHD from settlements to towns in Greenland. The difference in rates of CHD in women were less apparent, with lower rates in Denmark than in towns in Greenland. These studies do not have sufficient specificity to identify omega-3 fatty acids as causal in reducing CHD, but are consistent with the hypothesis that they are.

Van Houwelingen et al. (Ref. 294) found that, while men from a high fish consumption group had higher concentrations of plasma phospholipid EPA and DHA than men from a low fish consumption group, there was no significant difference in collagen-induced platelet aggregation, cutaneous bleeding time, ATP-release in whole blood, or platelet number between the two groups. This study suggests that the outcome measures found commonly to be affected in clinical studies may not be related to consumption of omega-3 fatty acids in the free-living population.

c. Intervention studies

There were no new prospective intervention studies measuring occurrence of heart attacks or CHD mortality.

2. Evidence relating omega-3 fatty acids to intermediate or surrogate markers of CHD (Table 2)

a. Atherosclerosis

i. Blood lipids

Through its own literature review, FDA has found another 34 studies not reviewed in the proposal that report data for serum cholesterol after consumption of fish containing omega-3 fatty acids or fish oil concentrated in omega-3 fatty acids. Among these, 25 found no change in blood cholesterol levels, 3 found an increase, and 6 found a decrease.

Studies among normal healthy subjects generally reported no change in total cholesterol (Refs. 168, 196, 202, 210, 217, 220, 235, 241, 253, 254, and 277), although none of these studies was controlled for nonspecific effects of the omega-3 fatty acids as polyunsaturated fats.

One study among normal subjects found that feeding a high fish diet did not change total cholesterol, unless combined with a low total fat and low saturated fat diet (Ref. 168). Another study (Ref. 301) reported decreased total

cholesterol after switching from a meat diet to a fish diet, but the fish diet had significantly less saturated fat than the meat diet. One study (Ref. 283) found a slight increase after 5.4 g EPA plus DHA/day from MaxEPA (with 30 percent saturated fatty acids), and one study (Ref. 224) found a slight reduction in total cholesterol after 2.7 g purified EPA/day, but neither study was placebo controlled for effects of polyunsaturated and saturated fat contained in the supplements.

Similarly, nearly all of the 17 studies on subjects in at-risk subpopulations, including all of the studies that controlled for PUFA's (Refs. 203, 209, 247, and 258), found no effect of supplemental omega-3 fatty acids on total cholesterol (except for a post hoc analysis of a subgroup in one study; (Ref. 209)). One study in diabetics (Ref. 252) found an increase in serum cholesterol, but the statistical significance of the result may have been due in part to a change in the opposite direction in the control group. One study among hyperlipidemics (Ref. 191) found decreased cholesterol after relatively high doses (4.6 to 6 g EPA plus DHA/day) but not after 3.6 g EPA plus DHA/day, and did not control for PUFA effects of the supplements. The other study that reported decreased cholesterol after supplemental omega-3 fatty acids (Ref. 268), similarly, found the effect after a high level (6 percent of calories, 16 to 21 g EPA plus DHA/day) and did not control for the polyunsaturated fat effects of the supplement.

These studies support the conclusion reached in the proposed rule, that among normal, healthy subjects there is no significant effect of omega-3 fatty acids from fish or fish oils on total serum cholesterol.

FDA concluded in the proposed rule that the best studies among normal subjects found no effect of fish oils on LDL cholesterol. All of the additional studies among normal healthy subjects obtained in FDA's updated literature search have reported no change in LDL cholesterol (Refs. 220, 253, 253, and 277).

One study (Ref. 224) reported that purified EPA produced a significant decrease in a subtraction of large, light LDL cholesterol (LDL₁), and a significant increase in small, dense LDL cholesterol (LDL₂), but FDA calculates no change for the sum of these two fractions of LDL cholesterol. Some clinical studies reviewed in the proposed rule (Refs. 1, 43, 53, and 129) described changes in the composition of LDL particle after consumption of fish oil.

The relative importance of various subfractions of LDL particles (and the associated composition of the particles), however, is still controversial. While Homma et al. (Ref. 224) suggest that large, light LDL are the fraction associated most closely with atherosclerosis, Austin et al. (Ref. 171) report that the phenotype of small, dense LDL is the fraction most closely related to increased CHD risk. The February 1992 NHLBI consensus development conference (Ref. 255) included among its recommendations for further research the identification of the atherogenic and anti-atherogenic subfractions that may be present in VLDL and HDL; the uncertainty about the relevance of changes in the amounts of subfractions of these two lipoproteins similarly applies to LDL.

In at risk populations, there have been some additional reports of increased LDL cholesterol after fish oil supplementation (Refs. 170, 191, and 251), a concern raised in the proposal. However, most studies have found LDL cholesterol not changed by fish oils (Refs. 174, 189, 205, 209, 219, 258, and 278). Moreover, each of the studies that used a polyunsaturated fat placebo control group found no change in LDL cholesterol (Refs. 203, 209, and 258).

Therefore, FDA concludes that these most recently reviewed studies support the conclusion reached in the proposed rule, that for the general population, there is no significant effect of omega-3 fatty acids on LDL cholesterol. The results of recent studies among at-risk subjects, however, are not in complete agreement with the conclusions in the proposed rule, and suggest that omega-3 fatty acids may not uniformly increase LDL cholesterol. Additional study is needed to determine the conditions under which LDL cholesterol is increased by omega-3 fatty acids.

Among more recent studies in normal healthy subjects found in FDA's updated literature review, about half have found no effect of fish oils or fish on HDL cholesterol (Refs. 202, 206, 217, 219 (after 1.25 and 2.5 g/day EPA plus DHA), 226 (after 1 and 3 g/day EPA plus DHA), 241, and 244), but about half have found increased HDL (Refs. 210, 219 (after 3.75 and 5 g/day EPA plus DHA), 220, 226 (after 6 g/day EPA plus DHA), 235, 253 (compared to baseline, significant compared to olive oil control), 278, 283, and 301), including a metabolic ward study that very carefully controlled for total fat and saturated fat intake (Ref. 253). Weintraub et al. (Ref. 298) found decreased HDL after fish oil compared to saturated fat diet.

FDA attempted to ascertain how those studies that reported an increase in HDL cholesterol after increased intake of omega-3 fatty acids differed from those studies in which no effect was found, however, there was no apparent difference between the studies that reported that omega-3 fatty acids reduced HDL cholesterol and those that reported no change. Most of the studies that found a change used supplements containing substantial amounts (e.g., 30 percent) of saturated fatty acids, raising the possibility that the saturated fatty acids in the supplements were responsible for the increase in HDL (Ref. 17). However, some supplements had low amounts of saturated fatty acids (Ref. 278) or saturated fat in the diet was specifically controlled (Ref. 235), and in one study the control diet was reported to have significantly more saturated fat than the fish diet (Ref. 301), so the saturated fat intake during omega-3 fatty acid supplementation cannot be the factor responsible for increased HDL.

The amounts of omega-3 fatty acids used in those studies that reported increased HDL tended to be high (e.g., more than 5 g EPA plus DHA/day), but some studies that found a change used lower amounts (Ref. 278) and some studies that used high amounts found no change (e.g., Ref. 241 (used 6.7 g EPA plus DHA/day) and 253 (used 8 g/day)). Some studies in which fish was fed, rather than fish oil, found an effect (Refs. 235 and 301), but others did not (Refs. 206 and 244). There was no systematic difference in sample sizes of the studies that found an effect and those that did not; seven of the negative studies reviewed in the proposed rule or in the present document had 30 or more subjects, compared to only one of the positive studies. Small studies (n = 10 or fewer) may not have observed a significant difference because of small sample size, but larger studies did not find a significant difference, even though some found a trend toward increased HDL after fish oil supplementation (Ref. 217).

Finally, the enrichment of plasma phospholipids with EPA and DHA tended to be higher for subjects in studies where increased HDL was found than that for subjects in the studies where no change in HDL was found, reflecting the tendency of higher doses to produce increased HDL. In particular, all studies in which the plasma phospholipid EPA value was 3.9 percent or more found increased HDL. However, the studies that fed the highest amounts of EPA but that did not find an effect on HDL did not report data for phospholipid EPA, so it is not clear whether high phospholipid EPA is

uniformly associated with increased HDL. Comparable results were found after inspection of data on phospholipid DHA after supplementation, however, because not all studies reported phospholipid fatty acid values, no conclusion can be drawn about the relationship between phospholipid DHA and HDL concentration. Notably, recent data suggest a direct correlation between plasma EPA and HDL, but an inverse relationship between plasma DHA and HDL (Refs. 177 and 178), underscoring the importance of reporting these data in future studies.

Among subjects with risk factors for CHD fewer reports found increased HDL (Refs. 191 (for type IV on SuperEPA only), 195, 203, 209 (for type lib), and 219) than found no change (Refs. 170, 174, 189, 191 (for type lib and type IV on MaxEPA), 209 (type IV), 224, 258, 263, 277, and 299).

Few studies have controlled for effects of PUFA's by giving a PUFA supplement. Two papers found no change in HDL in normal subjects fed fish oil as Promega (Ref. 73) or MaxEPA (Ref. 166) compared to wheat-germ oil or safflower oil (Refs. 73 and 166, respectively). Cobiac et al. (Ref. 20) reported increased HDL for mildly hypertensive subjects fed salmon and sardines in sild oil compared to those given a safflower-olive oil mix, but in comparable subjects, Meland et al. (Ref. 247) found no change in HDL cholesterol after MaxEPA fish oil compared to when the subjects were given a corn-olive oil mix. Very recent results, also for a mildly hypertensive population, found increased HDL after either, ethyl esters of EPA and DHA, or after corn oil (Ref. 177). Thus, for normal and hypertensive subjects, the change in HDL appears to not be a specific effect of omega-3 fatty acids, but may be related nonspecifically to increased PUFA's, either omega-3 fatty acids or omega-6 fatty acids.

In contrast, there are two reports of increased HDL cholesterol in subjects with type lib hyperlipidemia fed fish oil compared to safflower oil (Ref. 166) or a corn-olive oil mix (Ref. 209), and one report of increased HDL in type IIa hyperlipidemics after fish oil or olive oil compared to corn oil (Ref. 286). Others found fish oil did not change HDL in type IV hyperlipidemics (Refs. 166 and 209) or patients with CHD (Ref. 258) compared to PUFA controls.

Therefore, at this time, FDA concludes that there is some evidence that omega-3 fatty acids, in some form and amount and in some selected populations, may increase HDL cholesterol, but that current data are ambiguous because the conditions

under which fish oils reliably increase (total) HDL cholesterol have not been established, either in a specific subpopulation, or in the general population.

When fractions of HDL cholesterol have been reported, an increase has generally been found in the HDL₂ fraction (Refs. 1, 9, 54, 148, 191, 202, 203, 220, 235, 251, and 286), with a comparable decrease in the HDL₃ fraction (Refs. 202, 235, 251, and 286). Interestingly, the two recent reports that failed to find increased HDL₂ both used esterified omega-3 fatty acids rather than the fish oil triglyceride (Refs. 191 and 224), although others using ethyl esters have found increased HDL₂ (Refs. 9 and 286).

These studies suggest that fish oils produce a shift within the HDL fractions toward a lipid-rich, and away from a protein-rich lipoprotein, as well as within the LDL fractions. This shift may occur whether or not there is any change in total HDL cholesterol. FDA noted (56 FR 60663 at 60669) that some studies among normal subjects found increases in the HDL₂ fraction of HDL cholesterol, and that these reports were the most promising changes in blood lipids. New studies published after the period covered in FDA's review of the literature, however, found that both HDL₂ and HDL₃ were correlated with reduced risk of MI (Refs. 185a and 287a), and the NHLBI consensus conference (Ref. 255) concluded that, "The current studies of HDL₂ and HDL₃ levels have not shown consistent associations with CHD." Therefore, data on changes in HDL subfractions after increased consumption of omega-3 fatty acids do not provide a sufficient basis for a health claim, because there is not significant scientific agreement that the endpoints are directly related to risk of CHD. If the risk of CHD becomes linked with particular subfractions of these lipoproteins, these findings in normal subjects may be of great importance.

However, FDA also notes that recently published data from a prospective study demonstrate an effect of aspirin consumption in reducing the incidence of first heart attacks among women (Ref. 243). Another study shows a relationship between spontaneous platelet aggregation in vitro and incidence of CHD (Ref. 288). Both studies were conducted in the general population and their results support the hypothesis that platelet aggregation is a useful marker for CHD risk in the general population. Additionally, preliminary data from the Caerphilly Collaborative Heart Disease Study (Ref. 302) supports a relationship between platelet aggregation and the incidence of

ischemic heart disease; final data from this study will be available in the near future. These recently published and forthcoming studies may provide the basis for significant scientific agreement regarding the use of platelet function as a surrogate marker for CHD risk among the general population.

ii. Vessel wall effects.

New human studies on the effects of omega-3 fatty acids on vessel wall effects were discussed in response to comments 35 through 37 of this document. A recent meta-analysis of studies on use of fish oils in the prevention of restenosis concluded that the most plausible interpretation of the results was that there was a small to moderate beneficial effect of fish oils, but that chance could not be ruled out as a cause of the results (Ref. 260). The authors noted a significant heterogeneity in the findings and concluded that data from a large clinical study are necessary to confirm their interpretation. No study of restenosis to date has compared fish oil to an alternate polyunsaturated oil to control for nonspecific effects of PUFA's.

b. Thrombosis and hemostasis

i. Bleeding times

A number of studies have reported data that show no significant effect of fish oils on standardized bleeding times (Refs. 179, 218, 253, 268, and 277). However, others have found a significant increase in bleeding time due to fish oil (Refs. 195, 219, 220, and 278) or salmon (Ref. 297) or have reported increased bleeding as a side effect of treatment (Refs. 189 and 295).

ii. Platelet aggregation.

Consistent with the literature previously reviewed, recent studies show that fish oil tends to decrease platelet aggregations to numerous stimuli including AA (Refs. 179 and 256), adenosine diphosphate (ADP) (Refs. 204, 256, and 297), collagen (Refs. 218, 241, 251, and 297), thrombin (Ref. 241), and PAF (Ref. 251). Only one of these studies controlled for effects due to PUFA's (Ref. 204). The importance of the polyunsaturated fat control is less critical for studies on platelet function than for studies on blood lipids, because nonomega-3 PUFA's (i.e., omega-6 fatty acids derived from plant oils) produce effects in the opposite direction in platelets as omega-3 fatty acids (whereas many of the blood lipid effects of these two classes of fatty acids are in the same direction). Thus, the effects of omega-3 fatty acids on platelet responsiveness are not likely to be produced by PUFA's in general.

The only new study among healthy subjects that reported no difference in responsiveness to ADP used EPA ethyl esters as the source of omega-3 fatty acids (Ref. 179). Furthermore, the data were not shown in this brief report, so it is not clear if there was a trend toward an effect that might not have been statistically significant due to small number of subjects (eight per group). Those studies in healthy subjects reviewed in the proposed rule that did not find statistically significant differences in platelet responsiveness to ADP did have trends in the direction of reduced responsiveness (Refs. 24 and 54).

Other studies found no effect of fish oils on platelet aggregation in response to collagen (Refs. 179, 256 and 277). Each of these studies had a relatively small number of subjects, and there was a trend toward decreased sensitivity toward collagen at a high dose of omega-3 fatty acids in one study (Ref. 277). However, in the recent metabolic ward study (Ref. 256) there was no trend toward decreased sensitivity toward collagen or thrombin. These findings contrast with the results described above (Refs. 218, 241, 251, and 297) and with studies in healthy subjects described in the proposed rule (Refs. 2, 24, 54, 86, 143, and 166).

Studies reporting no effect of fish oils on PAF or AA-induced platelet aggregation (Refs. 179 and 218) may not have had sufficient power to find a statistically significant difference; where the data were reported there was a trend toward decreased sensitivity for both agents (Ref. 218).

iii. Platelet adhesion

A provocative study by Li and Steiner (Ref. 234) showed a 60-percent decrease in the extent to which platelets prepared from subjects fed fish oils adhered to substrates in a laminar flow chamber. The high flow rates used in this experiment showed that the change in adhesiveness of the platelets was due to changes on the platelet surface, and not due to a difference in the amount of material released from platelets that subsequently caused adhesion (i.e., AA). Also, a dose-response relationship was observed, and the time to return to pre-fish oil adhesion values was related to the amount consumed.

However, another study found no effect on fish oils on in vitro platelet adhesion to everted rabbit aorta, although there was a trend toward increased adhesion after 2 and 4 weeks of supplementation (Ref. 264). The reperfusion assay used in this study does not distinguish platelet membrane effects from effects mediated by

substances released from platelets. Neither of these studies used a nonomega-3 PUFA control.

iv. Regulators of bleeding

Two recent studies in normal subjects have reported that omega-3 fatty acids have no effect on the clotting protein fibrinogen (Refs. 183 and 210), although in one of these studies a large supplement of vitamin E was associated with a decrease (Ref. 210). An uncontrolled study in normal subjects found a decrease in fibrinogen after fish oil supplementation (Ref. 278).

Studies on subjects at risk for CHD have reported no change (Ref. 203), a decrease (Refs. 276 and 277), and an increase in fibrinogen (Ref. 287). In agreement with its tentative conclusion in the proposed rule, PDA finds that the data on the effects of omega-3 fatty acids on fibrinogen level are ambiguous, because they do not distinguish effects due to PUFA's from effects specific to omega-3 fatty acids.

Plasminogen is an enzyme that dissolves clots. Plasminogen activator is a substance that increases clot dissolving; plasminogen activator is specifically inhibited by another substance, the PAI-1. Thus, a high level of PAI-1 decreases the capability to dissolve clots.

Three recent studies reported increased concentrations of PAI-1 after fish oil supplementation (Refs. 254, 278, and 287), which would appear inconsistent with a clot-dissolving effect of fish oil. Two of those investigators also found no change in the amount of plasminogen activator (t-PA) after supplemental fish oil (Refs. 254 and 287) including one who used a very specific immunologic assay (Ref. 254), suggesting that fish oils do not increase clot dissolution by increasing the amount of this protein. The third group however, found an increase in the activity of tissue plasminogen activator (Ref. 277), which suggests that fish oils might increase clot dissolution by a different mechanism than affecting the amount of activator. Another group found no effect of cod liver oil on t-PA activity or fibrinolysis measured directly (Ref. 216). These reports are in contradiction to a report of increased fibrinolytic activity after a fish or fish plus fish oil diet (Ref. 183). FDA has not been able to find a reason for this rather marked contradiction. Therefore, in agreement with the conclusion in its proposed rule, FDA finds there is no clear relationship between omega-3 fatty acids and factors involved in dissolving blood clots, or clot dissolution activity.

Numerous investigators (Refs. 174, 191, 210, 220, 235, 241, and 279) have

recently reported that fish oils do not affect the concentration of Lp(a), a lipoprotein correlated with the risk of CHD. One investigator reported that very high levels of fish oils (9 g EPA plus DHA/day) gave a trend toward lower values, but the response may have been due to the PUFA's (Ref. 279). One study reported no effect overall of fish oils on Lp(a) among hypertriglyceridemics, but Lp(a) was reduced in those whose initial values were high (Ref. 174). On the basis of these reports and those reviewed in the proposed rule, FDA concludes that omega-3 fatty acids do not affect the risk of CHD by lowering Lp(a).

v. Blood pressure

Most of the studies not reviewed in the proposed rule that report data on blood pressure after consumption of fish oils have not found a significant change. One study of 50 elderly, healthy subjects reported that fish oils in combination with a salt-restricted diet decreased both systolic and diastolic blood pressure, but that fish oil alone had no effect (Ref. 190). There was a reduction in blood pressure during the run-in period, when the polyunsaturated fat placebo, sunflower oil, was fed.

Most studies on subjects with mild hypertension also have reported no change (Refs. 247, 277, and 289), including one large, randomized, placebo-controlled, multicenter trial of various behavioral changes and dietary supplements (Ref. 289). One study in hypertensives found reduced systolic and diastolic blood pressure comparable to reductions after the hypertension medication propranolol (Ref. 285), and in some cases the combined treatment of fish oil plus propranolol gave a greater decrease than either treatment alone. This study was controlled by olive oil (which is predominantly monounsaturated fatty acids), and therefore does not distinguish effects of omega-3 fatty acids from other PUFA's. Another double-blind randomized, placebo-controlled study in hypertensives whose blood pressures were maintained by medications found comparable blood pressure lowering compared to pretreatment values by fish oil or olive oil placebo (Ref. 299):

One uncontrolled study among hyperlipidemics also found reduced systolic and diastolic blood pressure (Ref. 263), but no effect was found in uncontrolled trials in subjects with end-stage renal disease (Ref. 207) or diabetics (Ref. 215). In a polyunsaturated fat (corn oil) controlled study on subjects with stable claudication (Ref. 203) fish oil and corn

oil both reduced diastolic blood pressure comparably, but systolic blood pressure was only reduced by the corn oil treatment

The results of these studies support the tentative conclusions reached in the proposed rule, that omega-3 fatty acids reduce blood pressure to a small degree in hypertensive people, but that it is not clear if there is any specific effect among normal subjects.

3. Other relevant information

a. Animal studies

Animal studies are especially important for studying effects of long-term consumption of omega-3 fatty acids, where there are few data from human intervention studies. The animal studies cited in the proposed rule related to the ability of omega-3 fatty acids to inhibit the development of atherosclerosis, an area not readily available for study in humans. A more complete discussion of the previously cited studies, with emphasis on those studies in nonhuman primates, is given in response to comment 47 of this document. Other recent animal studies cited in the comments or found during FDA's updated literature search that provide data on the development of atherosclerosis (where atherosclerosis is measured directly) are reviewed here. Also reviewed are studies on effects of omega-3 fatty acids during experimental ischemia, obviously not available for human study.

i. Atherosclerosis

One recent study in rabbits found less atherosclerosis in fish oil-supplemented animals, but there was no control for PUFA's, and the fish oil-treated animals also had reduced serum cholesterol (Ref. 192). Because humans do not have reduced serum cholesterol after fish oil consumption, these results are of questionable relevance to humans. Furthermore, the effect cannot be attributed specifically to omega-3 fatty acids rather than to polyunsaturated fats in general.

Fish oil feeding has also been associated with reduced binding of LDL to the blood vessel endothelium in monkeys (Ref. 193), and purified EPA ethyl ester was reported to reduce susceptibility of LDL to oxidation (Ref. 273), but these studies did not control for PUFA's. The antioxidant levels in the diets with respect to the amount of omega-3 fatty acids may be as important in determining whether or not there is any effect of omega-3 fatty acids on the oxidation of LDL.

Three recent papers describe effects of fish oils fed before surgical grafting of a

vein into an artery, a procedure associated with an accelerated development of atherosclerosis. Two papers (Refs. 275 and 303) each used a polyunsaturated fat control and studied fish oil effects after vein allografts in animals treated with the immunosuppressant cyclosporin. In one study (Ref. 303), six groups of rabbits received one of three amounts of fish oil (giving 29, 87 and 174 mg EPA plus DHA/kg, respectively, similar amounts to those used in most human studies) or comparable amounts of safflower oil. In this study, safflower oil was more effective at reducing cholesterol than fish oil, and there was a trend toward more protection from atherosclerosis in the safflower oil-fed group. In the other study (Ref. 275), rats received, in addition to cyclosporin, either fish oil (containing 210 mg EPA plus DHA/kg), or safflower oil with aspirin, or safflower oil only. The fish oil group had remarkably less atherosclerosis than the other two groups. The contradictory results in these two studies, both of which used the same model of vein allografts with cyclosporin immunosuppression and the same polyunsaturated fat control, may be related to dose and species differences.

A third study of vein allografts in dogs (Ref. 274) found significantly less atherosclerosis in fish oil-fed animals either fed the fish oil alone or in combination with aspirin or a thromboxane synthetase inhibitor. Other animals were treated with aspirin only or a thromboxane synthetase inhibitor only. There was no difference among groups for blood lipids, platelet function or eicosanoid metabolism. This study suggests that mechanisms of atherosclerosis other than those involving blood lipids and platelet function may be affected by omega-3 fatty acids.

These animal models are most relevant to comparable surgical procedures or other invasive procedures (e.g., angioplasty) that would be expected to activate platelets in humans. Use of omega-3 fatty acids in these settings is a drug usage, but provides information on the extent to which omega-3 fatty acids may modify platelet response *in vivo*. The very different results of omega-3 fatty acids in modifying the response to vein allografts in immune-suppressed animals indicates that the actions of omega-3 fatty acids in these settings are not yet well established.

ii. Response to ischemia

One major line of research on omega-3 fatty acids in animals is experimental ischemia (deficiency of blood flow to

the heart). Force et al (Ref. 199) found that rats fed a diet containing a high amount of fish oil (20 percent of the diet.) for 6 to 12 weeks had much greater blood flow once the occlusion was removed than rats fed diets enriched in corn oil or lard. There were no differences among diet groups in the amount of tissue damaged by ischemia. Increased blood flow after ischemia has also been reported in a pig model (Ref. 221). This study did not control for polyunsaturated fat and used a lower amount of omega-3 fatty acids, and the differences in blood flow were not as pronounced as in the Force study. Another study found evidence of less tissue damage during reperfusion when rats had been fed a diet with 1.2 percent fish oil compared to other rats fed the same level of corn oil (Ref. 223). Another study in yet a third animal species (Ref. 230) showed that the functional capillary density was preserved during reperfusion in hamsters fed 5 percent fish oil for 4 weeks prior to experimental ischemia.

A reperfusion study in dogs determined the effects of fish oil on the duration of time needed for drug-induced reperfusion following an electrically induced blockage, and on the occurrence of spontaneous reocclusion in the reopened vessel (Ref. 232). High amounts of fish oil (one-third total calories) for 3 weeks before the surgery resulted in a shorter time needed for the drug-induced reperfusion, but did not affect the time necessary for the electrically mediated occlusion to occur, the occurrence of second occlusion, or the time it took for the second occlusions to appear. This study did not have a polyunsaturated fat control, and even at the high intake only a modest effect of omega-3 fatty acids on platelet function was seen, that being primarily an enhancement of the effects of the fibrinolytic drug.

Another possible consequence of ischemia is arrhythmia, when the heart fails to maintain its normal rhythmic beating. The effects of fish oils on arrhythmia in monkeys are discussed in response to comment 39 of this document. Similarly, data have been reported for experimental ischemia in rats that show that, both fish oil and sunflower oil reduced the occurrence of arrhythmia during occlusion and reperfusion compared to a saturated fat diet (Ref. 246). Another study, done on isolated, cultured rat heart cells (myocytes) showed that EPA, but not AA, prevented a known toxin (ouabain) from disturbing the rhythmic contractions and killing the cells (Ref. 212). The effective amount of EPA was so low that the mode of action was

proposed to be due to production of an active metabolite, rather than due to direct effects of EPA on the cell membranes. This study suggests a specific effect of EPA in stabilizing the heart myocytes during stress. Prevention of arrhythmia by stabilization of these heart cells has been proposed as a mechanism by which omega-3 fatty acids may increase the chances of survival following a heart attack as reported in the Dart study (Ref. 16).

These studies indicate that, in various animal models, dietary fish oils promote greater reestablishment of blood flow in heart tissues following a transient block as occurs in an acute heart attack. Importantly, the results are consistent across many animal species, and in some cases have been shown to be specific for omega-3 fatty acids rather than simply due to any PUFA. Finally, the experimental designs included coronary occlusions in otherwise healthy animals who were not suffering from heart disease, a model relevant for use of omega-3 fatty acids in reducing the risk of CHD rather than in therapy for persons with preexisting heart disease. The studies remain limited in that ischemia was produced by an acute blockage produced by mechanical or electrical means rather than by chronic dietary means, and the response to these different types of block may not be the same.

Other studies have attempted to learn the mechanisms by which the platelet-vessel wall interactions are modified by omega-3 fatty acids. One study (Ref. 240) found that aortas from rats fed fish oil or corn oil did not contract as much in response to agents that cause contraction as aortas from rats fed beef tallow (saturated fat). This was true both before and after oxygen deprivation. The aortas from fish oil-fed rats were more responsive to one of three tested chemical relaxers than aortas from corn oil-fed or beef tallow-fed rats. Another study found, that EPA potentiated the release of an EDRF (Ref. 181), but the effect was thought to be related to the unsaturation of the EPA, because the experiments were carried out in the presence of inhibitors of EPA metabolism.

One research group has recently shown that leukotrienes, chemicals produced from AA, are important in the tissue injury that accompanies reperfusion (Refs. 230 and 232). Since EPA competes with AA for the enzyme that makes leukotrienes from AA, EPA could potentially reduce the amount of leukotriene formed from AA. This same group has shown that leukotrienes promote the adhesion of leukocytes to the vessel wall (Ref. 231), and that

feeding hamsters fish oil at 5 percent of the diet for 4 weeks greatly reduced (over 60 percent) the adhesion of leukocytes to the vessel wall (Ref. 233). The reduced adhesion could be relevant for both the conditions during which atherosclerosis develops (indeed, the stimulus used to elicit leukocyte adhesion was oxidized LDL, a candidate for promoting atherosclerosis in humans), and the acute response to coronary ischemia.

These animal data suggest mechanisms by which omega-3 fatty acids could affect the development of atherosclerosis or the response of heart tissue after a transient occlusion of its blood flow. Both modes of action could make important contributions to the risk of CHD and, therefore, merit additional study. The reperfusion studies and the myocyte toxicity study have demonstrated specificity of the effect as to omega-3 fatty acids. However, the increase in reperfusion volume is not sufficient to ensure a reduced risk of CHD death. Omega-3 fatty acids may not affect the extent of tissue damaged during an occlusion, or the tendency for a second, spontaneous occlusion. Additionally, omega-3 fatty acids may not affect tissue vulnerability during reperfusion. Those studies where CHD deaths or second occlusions have been recorded used large amounts of fish oils, and do not indicate whether amounts of omega-3 fatty acids found in the diet would have the same effects. Thus, there are many possible avenues suggested by these animal studies for beneficial effects of omega-3 fatty acids on the development of CHD, but there are also important limitations in the study designs and models used that prevent drawing conclusions from these data about the importance of omega-3 fatty acids in reducing the risk of human CHD.

b. Safety concerns

i. Diabetes

Three additional papers (Refs. 170, 222, and 304) and one major review (Ref. 238) on the effects of fish oils in diabetics were published after the time period reviewed in the proposal. All three new studies found increased LDL cholesterol after fish oil consumption in type II diabetics. However, effects of fasting glucose varied, with no change (Ref. 170), a transient increase (Ref. 222) or an increase (Ref. 304) reported. Although fasting insulin concentration was unchanged after fish oil (Refs. 170- and 304), postprandial, insulin response usually, but not always (Ref. 170), has been reported as reduced (Refs. 238 and 304).

These effects of fish oils on blood glucose appear to depend on the amount of fish oils fed. One study found no change in fasting blood glucose levels among type II diabetics treated with 3.0 g/day EPA plus DHA for 2 weeks (Ref. 170). Two other studies that used 3 or 2.7 g/day EPA plus DHA for 6 and 8 weeks (Refs. 222 and 79) only found transient increases in blood glucose halfway through their respective supplementation periods. A fifth study (Ref. 12) that used 3.0 g/day EPA plus DHA for 3 weeks found comparable increases in fasting blood glucose when either fish oil or safflower oil was fed, so the increase cannot be attributed specifically to the omega-3 fatty acids. Similarly, Vessby and Boberg (Ref. 157) fed 3 g/day EPA plus DHA and did not find a difference in fasting glucose or glycosylated hemoglobin after fish oil supplementation compared to baseline; they did find a significant difference compared to the olive oil treatment that produced changes in the opposite direction from fish oil. Studies on type II diabetics that reported increased glucose used higher amounts (4.5 to 8 g/day) of omega-3 fatty acids (Refs. 52, 55, 128, and 304). Thus, FDA concludes that glycemic control among diabetics remains a valid safety concern, but notes that restriction of the amount of supplemental omega-3 fatty acids may suitably address this concern.

ii. Increase in LDL cholesterol

Many studies published after 1987 with data for LDL or apoB report increased LDL cholesterol or apoB after fish oils, in hypercholesterolemic or hypertriglyceridemic subjects (Refs. 1, 26, 32, 55, 60, 61, 63, 73, 75, 94, 114, 119, 120, 129, 146, and 166). Virtually all the studies with 10 or more subjects supplemented with 5 g/day EPA plus DHA or more found increased LDL. Some studies on normal subjects (all of which were reviewed in the proposed rule) also report increased LDL or apoB after fish oil consumption (Refs. 54, 127, and 156). Many studies that found no effect may not have had sufficient sample size to detect a difference due to treatment. FDA concludes that increased LDL cholesterol among some populations already at increased risk of CHD remains a valid safety concern, but because most reports of increased LDL are in studies where large amounts of fish oils are given, it is possible that restriction of the amount of supplemental omega-3 fatty acids and/or changes in the fatty acid composition of omega-3 fatty acid supplements may suitably address this concern.

III. Overall Summary and Conclusions

FDA concludes that there is some evidence that supports a relationship between omega-3 fatty acids and CHD, but that the totality of scientific evidence available at this time does not provide an adequate basis for a health claim. In some cases, there is not significant scientific agreement that the changes that are specific to omega-3 fatty acids will reduce the risk of CHD. In other cases, the data do not demonstrate that the effect is specifically due to the omega-3 fatty acids and not due to other dietary variables. For yet other cases, the data are ambiguous because effects of omega-3 fatty acids are not consistently observed, which suggests that other variables are important in determining whether or not an effect is seen. Therefore, the agency does not consider the evidence sufficiently strong to draw a firm conclusion about the relationship between omega-3 fatty acids and risk of CHD, and therefore is not authorizing the claim at this time.

In the course of developing this regulation, FDA has identified some areas where greater agreement is needed that the effects produced by omega-3 fatty acids are directly related to the risk of CHD. Many surrogate markers have been hypothesized, on the basis of limited evidence, to be related to specific diseases, including CHD, but few have withstood the continued scrutiny of scientific investigation. Also, some markers may have scientific validity, but may not be applicable for use in the general population, because of technical limitations. Thus, FDA asserts that only when a surrogate marker for a disease has been accepted as a risk factor for the general population, as indicated by a statement by an unbiased, nationally representative authoritative scientific or medical body, can the agency authorize a health claim based on the relationship of a nutrient to the surrogate marker of the disease. Examples of potential surrogate measures for which validation is needed are in vitro platelet aggregation, in vitro platelet adhesion, elevated fasting triglycerides, postprandial triglycerides (recently considered at the NHLBI consensus development conference. Ref. 255), and subtractions of LDL and/or HDL.

In some cases additional research is needed to determine whether hypothesized subpopulations, e.g., those with high LDL:HDL ratio and high triglycerides, are at increased risk of disease. The pronounced triglyceride lowering effects of omega-3 fatty acids

might well have a protective effect against CHD in such a subpopulation.

There are other areas where additional research is needed to show, for agreed endpoints, that the effects are consistently produced, or are specifically due to omega-3 fatty acids. These areas require additional data to establish that the effect of omega-3 fatty acids is specific, or to further define the conditions under which omega-3 fatty acids have their effects. For example, data are needed to show a reduction in MI or CHD mortality among individuals fed supplemental omega-3 fatty acids (specifically) compared to a group fed omega-6 PUFA'S. The critical failing of some recent studies associating omega-3 fatty acids and CHD is that specificity was not obtained. Future studies should carefully control for known confounders, particularly dietary variables.

Finally, the available data suggest that some set of conditions or population may exist for which omega-3 fatty acids will increase HDL. Additional research should be able to define the conditions under which omega-3 fatty acids have this effect.

Interested parties may choose to petition the agency for approval of other health claims about omega-3 fatty acids. For example, additional data may be developed to support an omega-3 fatty acids/hypertension health claim petition. Because the blood pressure-lowering effect of omega-3 fatty acids appears most marked against a background of very low dietary intakes of omega-3 fatty acids, the role of omega-3 fatty acids in the total diet would need clarification before such a petition could be adequately supported. Similarly, limitations of the effects of omega-3 fatty acids on the magnitude and duration of change in blood pressure, the quantitative amounts of omega-3 fatty acids required for the effects, and characterization of the sensitive subpopulation would require discussion in a petition. A petition should also address apparent conflicting pieces of information, e.g., high blood pressure among populations that have high intakes of omega-3 fatty acids. Safety concerns raised in this final rule will, of course, require resolution prior to the authorization of any petitioned claim.

IV. Decision Not to Authorize a Health Claim Relating Ingestion of Omega-3 Fatty Acids to Reduced Risk of CHD

In evaluating the scientific evidence, FDA considered: (1) The strength of the association of omega-3 fatty acids with CHD or surrogate markers for CHD, (2) the consistency of findings among the

many studies, (3) the specificity of the outcome to omega-3 fatty acids, (4) the presence or absence of a dose-response relationship, and (5) the biologic plausibility of an association. FDA has determined that there is inadequate evidence to show that increased consumption of omega-3 fatty acids will reduce the risk of CHD.

FDA sought to determine whether there was significant scientific agreement among qualified experts that the totality of publicly available scientific evidence supported the claim that omega-3 fatty acids reduce the risk of heart disease. FDA reviewed the position taken in numerous Federal Government reports and other authoritative scientific reports and evaluated the publicly available scientific evidence that has become available since those reports were written. The decision to deny a health claim is based on the conclusions reached following review of the following sources of information: (1) "The Surgeon General's Report on Nutrition and Health," (2) the National Research Council's "Diet and Health: Implications for Reducing Chronic Disease Risk," and (3) the National Cholesterol Education Program's Report of the Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults. Each of these reports concluded that there was inadequate evidence of a relationship between consumption of omega-3 fatty acids and CHD. FDA has reviewed again all of the relevant cross-sectional data from which the hypothesized relationship between omega-3 fatty acids and CHD originated, and all clinical intervention data published since these Federal Government and other authoritative reports were issued to determine whether the additional evidence is adequate to support a health claim for omega-3 fatty acids.

The LSRO report reached a different conclusion from the other authoritative reports by finding a relationship between omega-3 fatty acids and CHD. The report used only selected evidence, and often did not distinguish effects specifically due to omega-3 fatty acids from effects due to PUFA's in general. The description of international epidemiologic findings of a relationship between fish consumption and CHD, similarly, was not shown to be specific to omega-3 fatty acids. In some instances, FDA disagreed with the interpretation of the studies reviewed by LSRO, or with LSRO's conclusions.

Finally, the LSRO report also based its conclusions about the usefulness of omega-3 fatty acids, in part, on changes in blood lipid parameters that are not

generally agreed to be risk factors for CHD. Therefore, FDA finds numerous reasons for not accepting all of the findings of the LSRO report. FDA's conclusions regarding the relationship between omega-3 fatty acids and CHD rely instead on FDA's independent review of the publicly available scientific information, and are consistent with the Federal Government and other comprehensive and authoritative reports except for the LSRO report.

The surveys, cross-sectional studies, and nonintervention prospective studies do not provide adequate support for a relationship between consumption of omega-3 fatty acids and CHD. Only a few studies found an association between fish intake and CHD, while others have found no association; thus, there was no consistency of findings. None of the studies that reported a relationship distinguished fish consumption from other factors associated with fish consumption, and therefore none demonstrates specificity. Even in those studies that reported a relationship between fish consumption and CHD, it is not clear that the observed effects were due to the omega-3 fatty acids in fish. Also, the omega-3 fatty acid content of the fish diet associated with reduced CHD in these studies was so low that the importance of omega-3 fatty acids is questionable, thus weakening the biologic plausibility of the relationship.

The data from intervention studies also do not establish a relationship between omega-3 fatty acids and risk of CHD. The most compelling type of evidence to support a diet-disease relationship is a prospective, double-blind, placebo-controlled intervention study, with CHD morbidity and mortality as endpoints. To date, there is only one such trial (Ref. 16). The results of that study showed that increased consumption of fish does not reduce the risk of a second heart attack but may reduce the risk that the attack will be fatal. This study provides evidence for a protective effect of fish consumption against second heart attacks. However, as with the nonintervention study data, this study did not provide evidence to attribute the benefit to omega-3 fatty acid intake rather than some other factor associated with fish consumption (specificity).

Less persuasive than prospective studies in which CHD per se is measured, but still very useful, are prospective clinical trials in which surrogate markers for CHD are measured. Recent studies have not found beneficial effects from omega-3 fatty acids on total cholesterol or LDL

cholesterol in normal, healthy persons, or among persons at risk for CHD. Numerous studies, including some large or multicenter studies, have reported these results, demonstrating consistency in the findings and providing the agency confidence that they were not spurious. The data on HDL cholesterol are ambiguous. There appear to be other factors in the dietary interventions besides the omega-3 fatty acids that determine whether or not supplementation with fish or fish oils raises HDL.

An increase in bleeding times and a decrease in platelet aggregation have been observed frequently, but not always, after supplemental omega-3 fatty acids in normal healthy individuals as well as in diseased persons. Additionally, there is evidence that platelet adhesion is reduced by omega-3 fatty acids. The effects of decreased platelet aggregation and platelet adhesion appear to be related to the intake of omega-3 fatty acids in a dose-response relationship. What has not been established, however, is that in vitro platelet aggregation or platelet adhesion are bona fide surrogate risk factors for CHD in the general population.

Omega-3 fatty acids have been shown to reduce blood pressure in hypertensive people to a small degree, which may bear on a relationship between omega-3 fatty acids and CHD. This effect was not of large magnitude, but it is specific to omega-3 fatty acids, it has been reported by a number of investigators, a dose response was found, and the effect is biologically plausible through at least two mechanisms. However, it has not been established that omega-3 fatty acids reduce blood pressure in normal subjects (lack of consistency, weak effect, absence of dose-response relationship). Additionally, it has not been demonstrated that the magnitude and duration of changes in blood pressure observed in short-term studies will persist during long-term consumption of omega-3 fatty acids, or that these changes result in a reduced risk of CHD.

Finally, the potential that omega-3 fatty acids may increase LDL cholesterol and/or apoB among diabetics and hyperlipidemics, and the potential that omega-3 fatty acids may worsen control of blood glucose in diabetics are significant safety concerns that must be addressed before a claim may be made that consumption of omega-3 fatty acids by the general population will reduce the risk of CHD.

In conclusion, there are numerous effects of omega-3 fatty acids that may

be related to the risk of CHD, e.g., reduction in fasting and postprandial triglycerides, reductions in platelet aggregation and adhesion, changes in the composition of lipoproteins.

However, at this time these endpoints are not generally agreed to be closely related to the risk of CHD. In other areas, additional data are needed to show that effects related to fish consumption are specifically due to the omega-3 fatty acids in the fish, and to define the conditions under which omega-3 fatty acids consistently increase HDL. These avenues may provide a reasonable basis for a future petition for a health claim relating omega-3 fatty acids to the risk of CHD.

V. Environmental Impact

The agency has determined under 21 CFR 25.24(a)(11) that this action is of a type that does not individually or cumulatively have a significant effect on the human environment. Therefore, neither an environmental assessment nor an environmental impact statement is required.

VI. Economic Impact

In its food labeling proposals of November 27, 1991 (56 FR 60366 et seq.), FDA stated that the food labeling reform initiative, taken as a whole, would have associated costs in excess of the \$100 million threshold that defines a major rule. Thus, in accordance with Executive Order 12291 and the Regulatory Flexibility Act (Pub. L. 96-354), FDA developed one comprehensive regulatory impact analysis (RIA) that presented the costs and benefits of all of the food labeling provisions taken together. That RIA was published in the *Federal Register* of November 27, 1991 (56 FR 60856), and along with the food labeling proposals, the agency requested comments on the RIA.

FDA has evaluated more than 300 comments that it received in response to the November 1991 RIA. FDA's discussion of these comments is contained in the agency's final RIA published elsewhere in this issue of the *Federal Register*. In addition, FDA will prepare a final regulatory flexibility analysis (RFA) subsequent to the publication of the food labeling final rules. The final RFA will be placed on file with the Dockets Management Branch (HFA-305), Food and Drug Administration, rm. 1-23, 12420 Parklawn Dr., Rockville, MD 20857, and a notice will be published in the *Federal Register* announcing its availability.

In the final RIA, FDA has concluded, based on its review of available data and

comments, that the overall food labeling reform initiative constitutes a major rule as defined by Executive Order 12291. Further, the agency has concluded that although the costs of complying with the new food labeling requirements are substantial, such costs are outweighed by the public health benefits that will be realized through the use of improved nutritional information provided by food labeling.

VII. References

The following references have been placed on display in the Dockets Management Branch (address above) and may be seen by interested persons between 9 a.m. and 4 p.m., Monday through Friday.

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List of Subjects in 21 CFR Part 101

Food labeling. Reporting and recordkeeping requirements.

Therefore, under the Federal Food, Drug, and Cosmetic Act and under authority delegated to the Commissioner of Food and Drugs, 21 CFR part 101 is amended as follows:

PART 101—FOOD LABELING

1. The authority citation for 21 CFR part 101 continues to read as follows:

Authority: Secs. 4, 5, 6 of the Fair Packaging and Labeling Act (15 U.S.C. 1453, 1454, 1455); secs. 201, 301, 402, 403, 409, 701 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 321, 331, 342, 343, 348, 371).

2. Section 101.71 is amended by adding new paragraph (f) to read as follows:

§ 101.71 Health claims: claims not authorized.

* * * * *

(f) Omega-3 fatty acids and coronary heart disease.

Dated: October 30, 1992.

David A. Kessler,

Commissioner of Food and Drugs.

Louis W. Sullivan,

Secretary of Health and Human Services.

Note: The following tables will not appear in the annual Code of Federal Regulations.

BILLING CODE 4160-01-F

TABLE 1
Epidemiological Studies: Omega 3 Fatty Acids and CHD

Study	Study Design	Subjects	Methods	Results	Comments
Beswick et al. (Ref. 167)	Dietary Counseling compared with survey data and platelet activity 6 months later. Men from Cardiff, Wales with history of myocardial infarction.	56 men, ages 36 to 71 (mean=58). 13 excluded for medication, 1 for inadequate blood sample. 18 smokers, 34 patients on cardiovascular medication, 19 on B blockers, 8 on antihypertensive drugs, and 24 on angina medication.	Questionnaire to estimate polyunsaturated to saturated fat ratio, percent calories from fat, and EPA intake. Platelet activity based on ADP added for aggregation of platelets in blood and plasma.	Significant inverse correlation between fat ratio and platelet activity. Nonsignificant trend for decreased platelet aggregation with increased EPA intake.	Study inconclusive on role of EPA in platelet activity. Small sample. In vitro assay not repeated for accuracy. Nutrient intake estimated. Health behaviors, medications, and other factors which may have influenced results not presented. Study population ill.
Bulliyya et al. (Ref. 185)	Dietary and serologic survey in Nellore and Chittoor districts of India.	100 individuals in fish consuming populations and 109 individuals in nonfish consuming populations.	Dietary survey. Total serum and HDL-cholesterol, TG, and phospholipid measured. Clotting and bleeding times observed. Age, weight, pulse rate, blood pressure tabulated.	Total serum cholesterol was lower in the coastal village (152.7 mg/dL) than in the nonfish consuming village (214.9 mg/dL). HDL-cholesterol was higher in the coastal village.	Dietary survey methods not presented in paper. Dietary intake was presented as correlational data. The potential confounding effect of other components of diet on cholesterol level warrants explanation. Lower total cholesterol level in the fish eating population was observed.
Bjerregaard and Dyerberg, (Ref. 176).	Analysis of CHD mortality rates, Greenland.	All deaths due to CHD in Greenland from 1968 to 1983.	A register of deaths by cause was developed using information from death certificates, parish registers, and civil registration records. The register was computerized and brought under the management of the Danish Board of Health from 1975 to 1983.	Age standardized mortality rates per 10,000 person-years for CHD in men in Greenland settlements (5.3) were half those of men in Denmark (10.0). There was an increasing rate of CHD from settlements to towns in Greenland. The difference in rates of CHD in women were less apparent, with lower rates in Denmark than in towns in Greenland.	Genetic protection, exercise and confounding factors such as tobacco use cannot be eliminated as factors in this observation. These data support earlier observations of lower CHD rates in male Greenlanders versus Danes, but not for females. Note, the 50 percent difference is approximately the same as reported by Kromhout for comparing men eating no fish and those eating approximately 30 g/day.

TABLE 1--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Bonaa et al. (Ref. 178)	Dietary and serologic survey of residents of Tromso, Norway	156 subjects selected subjects from a survey population of 21,826 for an intervention trial.	Food consumption questionnaire, with separate questions for fatty and lean fish, also alcohol. Also, 2 unannounced 24-hour diet recalls. Analysis of covariance and multiple linear regression.	Fish consumption was inversely correlated with TG's, but no significant correlations between fish and cholesterol or apoprotein measures. EPA correlated with HDL until TG's were controlled. EPA correlated with TG even after HDL was controlled. DHA inversely correlated with HDL and apoA ₁	Divergent relationships between EPA and HDL, and DHA and HDL, may explain discrepancies in the literature regarding the effect of various supplements on HDL
Gerasimova et al. (Ref. 205)	Dietary and serologic survey of residents of Moscow and the Chukot peninsula.	Randomly selected men; Moscow n= 650 Chukot n= 261.	HDL by ultracentrifugation. A subsample was used for HDL phospholipids and apoproteins. 24-hour recall for dietary data.	Chukot residents had ↓ cholesterol, TG, LDL; ↑ HDL, ↑ consumption of omega-3 fatty acids, plasma lipid EPA.	Correlation between increased consumption of omega-3 fatty acids and serologic measures is consistent with the hypothesis of a relationship between omega-3 fatty acids and CHD, but many other dietary and behavioral factors could also be correlated and were not examined in this survey.
Innis et al. (Ref. 225)	Dietary and serologic survey.	Sample was selected as part of an unrelated dietary survey. 185 Canadian Inuit and 24 Vancouver residents.	Phospholipid fatty acid analysis.	Mean chain length and unsaturation index of the lipids in the two populations was very similar. The Inuit had greater EPA and lower AA than the Vancouver population. NS cholesterol	Observational data. Supports a dietary origin of phospholipids. Not directly relevant to CHD.

TABLE 1—CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Popeski et al. (Ref. 266)	Retrospective survey of pregnancy induced hypertension in Inuit women and diet survey of women in these communities	Hypertension study: 300 medical records from Inuit women in 7 communities in the Northwest Territories. Diet survey 27 Inuit women in the 7 communities above.	Retrospective study: Blood pressure measurements 6 hours before delivery, incidence of pregnancy induced hypertension. Diet survey: Reported diet, hunter interviews, cord serum phospholipid from 16 infants born in 6 months.	Communities with higher marine oil consumption have significantly lower diastolic pressure in their women in the last 6 hours of pregnancy. Pregnancy associated hypertension was 2.6 times more common in communities with low fish consumption.	Ecologic data. Generates hypothesis for a relationship between the consumption of fish and blood pressure during pregnancy. Prospective study or clinical trial of diet and pregnancy needed.
Seidelin et al. (Ref. 281)	Correlation study of adipose tissue fatty acids and extent of coronary artery stenosis.	40 consecutive autopsies from subjects age 52 to 90 years.	Coronary artery disease was quantified by semi automatic image analysis. Degree of stenosis was used to divide subjects into three groups for correlations. Umbilical adipose lipids were extracted with methanol-chloroform, trans-methylated esters by gas chromatography.	No correlation between extent of stenosis and 16:10, 18:0, 18:2n-6, 16:1n-9 or 18:1n-9, but a significant inverse correlation with 22:6n-3. Stenosis correlated with extent of body weight.	Limited data are presented for a limited number of subjects, i.e., no data for other fatty acids of interest are presented, e.g., EPA, AA.
Van Houwelingen et al. (Ref. 294)	Sample of clinical parameters from 40 healthy men selected from cohort in longitudinal study, Zutphen, Holland.	Men in low (n=15) and high (n=25) fish consumption groups. Low consumption group ate an average of 2 g fish/day while high consumption group ate an average of 33 g fish/day.	Individuals in this study where interviewed 4 times in 25 years using cross-check dietary methods for habitual fish consumption. Of 79 men selected for the study, 40 completed it. Blood collected for fatty acid composition, PAI activity, collagen-induced platelet aggregation, ATP release, and TXB ₂ .	There was significantly higher serum phospholipid concentration of omega-3 fatty acids EPA and DHA acid and no significant difference in collagen-induced platelet aggregation, cutaneous bleeding time, ATP-release in whole blood or platelet number between the two groups.	Small same reflects inconsistency of fish consumption over time. Dose of omega-3 in high fish consumption group lower than in most clinical trials.

TABLE 1--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Van Houwelingen et al. (Ref. 293).	Sample of clinical parameters from 61 elderly male volunteers from longitudinal cohort. Zutphen, Holland.	61 healthy elderly male volunteers ages 67 to 82. Lowest quartile of fish consumption ate 0 g/day. Highest quartile ate 27 g/day.	Cross-check dietary history was taken to assess the habitual intake of polyunsaturated fatty acids. Blood was collected under controlled conditions for measurement of phospholipids, TG's, and cholesterol esters.	Dietary history seemed to correlate with serum linoleic acid. The correlation between dietary history using the cross check method for fish consumption and serum level of fish-related fatty acids, however, seems less reliable.	The discrepancy between dietary history and serum level of fish-related fatty acid may be the result of large variation in the level of these fatty acids in the same foods.

TABLE 2
Intervention Trials of Omega-3 Fatty Acids and CHD

Study	Study Design	Subjects	Methods	Results	Comments
Agren et al. (Ref. 168)	Randomized parallel trial of fish, fish plus reduced fat (especially saturated fat) or usual diet (one fish meal per 2 weeks) for 15 weeks.	62 Normal, healthy female students.	Plasma lipids and lipoproteins at baseline, 7 and 15 weeks.	Controls: NS TG, cholesterol, apoA ₁ , apoB. Fish eaters: NS versus baseline TG, cholesterol, apoB; ↓ apoA ₁ ; ↓ TG versus controls. Fish plus low fat: ↓ TG, cholesterol, apoA ₁ , apoB.	This study shows the importance of the balance of the diet, particularly regarding saturated fat, in determining the blood lipid response to omega-3 fatty acids. Changes were slight unless a low fat low saturated fat diet was used. Study would have been stronger with a control group eating a low fat low saturated fat intake to the nonfish control.
Annuzzi et al. (Ref. 170).	Randomized double-blind crossover trial of 10 g fish oil/day (3.0 g EPA plus DHA, MaxEPA) versus olive, no wash out, 2 weeks each treatment.	Eight female NIDDM, without liver, kidney or any other disease known to influence lipid and/or carbohydrate metabolism.	One of the patients was treated by diet only; other on Glibenclamide (4) and metformin (3). The patients were under dietary control and they were in the metabolic ward.	↓ TG, VLDL; ↑ LDL; NS cholesterol, HDL FFA; NS LDL-TG, HDL-TG; NS fasting glucose, average glucose insulin response, sensitivity.	Duration period was very short and no fatty acid analysis on neither fish oil nor olive oil diet. There was no washout period between cross-over of the study.
Bairati et al. (Ref. 172)	Randomized, double-blind placebo-controlled trial of 15 g fish oil/day (MaxEPA, 4.5 g EPA plus DHA) versus olive oil from 3 weeks pre angioplasty to 6 months; concurrent aspirin.	119 subjects undergoing first, successful, computer quantified percutaneous transluminal coronary angioplasty, evaluated also at 6 months.	Angiographic assessment, quantified by computer. diet by food frequency. Restenosis defined in four was for analysis.	By three of four definitions of restenosis, and multivariate analysis to control for exclusions, fish oil reduced restenosis. NS ECG evidence of myocardial ischemia, but trend toward ↓ fish oil, also on exercise testing. Significant difference also according to dietary omega-3 fatty acids (highest, middle versus lowest tertile) although highest tertile intake was only 0.15 g/day, and dietary odds ratio comparable to fish oil odds ratio.	Comparable compliance. Olive oil control doesn't control for polyunsaturated fatty acids. The comparable magnitude of the effects of dietary omega-3 fatty acids and fish oil supplementation suggest long-term, low dose effects are as strong as short-term, larger amounts. No associations of restenosis with other dietary variables, total fat, classes of fat, cholesterol, or dietary seafood. Differences between groups use of blood pressure medications (see Bairati et al. Ref. 173)) was not discussed.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Bairati et al. (Ref. 173).	Randomized double-blind, placebo-controlled trial with 15 g/day MaxEPA (4.5 g EPA plus DHA) versus olive oil.	125 patients undergoing first percutaneous transluminal coronary angioplasty, evaluated also at 6 months.	Recumbent blood pressure, blood lipids by commercial kits.	Blood pressure increased in the olive oil control group, and was unchanged in the fish oil group, possibly because greater number of patients in the olive oil group discontinued concurrent blood pressure medications. Fish oil ↓ TG's, NS in cholesterol, LDL or HDL versus control, but the change from baseline was different between groups with ↑ LDL and HDL in fish oil.	Multiple linear regression analysis used to control use of blood pressure medications reduced differences in LDL and HDL to borderline significance ($p=0.06$), and inclusion of TG resulted in NS change in HDL.
Beil et al. (Ref. 174)	Randomized double-blind trial with 10.5 g/day fish oil (3.15 g EPA plus DHA, MaxEPA) versus 5.25 g/day fish oil plus 5.25 g oleic acid (low fish oil) versus 10.5 g oleic acid (placebo) 6 weeks.	30 Patients with primary hypertriglyceridemia. Patients off lipid lowering drugs for 6 weeks, no beta blockers, diuretics or hormones. Fat restricted diet (30 percent, polyunsaturated fat: saturated fat ratio 1.5:1 cholesterol <250 mg/day).	Lipids by standard methods, Lp(a) by commercial kit.	↓ TG in high fish oil group versus placebo; NS cholesterol, LDL, HDL apoB; NS Lp(a), post-hoc analysis shows ↓ Lp(a) in those initially greater than 10 mg/dL.	Although randomized, there were large differences in initial Lp(a) levels, with only 1 of 10 in the placebo group over 6 mg/dL versus 6 and 8 of 10 in the low and high fish oil groups, respectively. Therefore, on the basis of Lp(a) the randomization was inadequate. Oleic acid does not control for polyunsaturated fatty acids.
Bhathena et al. (Ref. 175)	Nonblinded, longitudinal design, 10 week run-in on placebo (15 g mixed fat); 10 weeks with 15 g fish oil (anchovy oil, 6.5 g EPA plus DHA); 8 weeks of 15 fish oil plus 200 mg vitamin E	40 Healthy females, no history of metabolic disease, no medication, no smoking.	Dietary for the placebo and test group was controlled, fish was eliminated from the menu. Forty percent of energy comes from dietary fat which was more than the level of current dietary guideline recommended.	Fish oil ↑ fasting glucose; ↓ TG, insulin, glucagon, GH, somatomedin-C; NS cholesterol, cortisol, dihydroepiandrosterone sulphate. Fish oil plus vitamin E gave no further change in glucose, TG, glucagon cortisol or cholesterol, but ↓ GH, insulin, increased somatomedin-C to placebo levels, and produced a ↓ in DHEA-S.	Unusual source and high level of omega-3 fatty acids. Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.

TABLE 2—CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Bonaa et al. (Ref. 178).	Randomized, double-blind placebo-controlled trial of 6 g/day Norek Hydro (4.5 g ethyl esters of EPA plus DHA) versus corn oil, 6 month observational run in, 10-week intervention.	146 healthy subjects.	Fasting blood samples at beginning and end of intervention, standard, commercial assays for lipids and apoproteins. Multiple linear regression.	Fish oil ↓ TG; In both groups NS cholesterol, LDL, apoB, ↑ HDL; ↑ apoA: in corn oil group. In fish oil group ↑ phospholipid EPA correlated with HDL, but not in corn oil group, whereas in corn oil group DHA was inversely correlated to HDL.	No change in dietary fat, alcohol or protein. Both groups had a small, significant weight gain. The divergent results underscore the need for studies on individual omega-3 fatty acids, that may help explain inconsistencies in results of fish oil effects on HDL.
Bordet et al. (Ref. 179).	Randomized, dose-response study to 300, 900, 2700 mg EPA (ethyl ester, MND-21, Mochida, Japan) 4 weeks plus 4-week washout.	32 healthy females.	Bleeding times by Simplate II.	NS platelet aggregation sensitivity to ADP, collagen, PAF; ↑ sensitivity to aze NS bleeding time.	Differences from other studies may be due to absence of DHA, and need for longer studies to allow DHA incorporation.
Brown and Roberts (Ref. 183).	Randomized 3 X 3 crossover of fish (0.6 g EPA plus DHA/day) versus fish plus fish oil (2.0 g EPA plus DHA/day) versus control (fish free) 6 weeks each with 6-week washout.	12 healthy females.	Clotting times on 2 samples taken 4 days apart at the end of each diet treatment. Fibrinolytic activity in pooled samples from each individual assayed by euglobulin activity versus fibrin.	↓ leukocytes on fish, fish plus fish oil diets. Platelet count ↓ on fish oil; NS fibrinogen, ↑ fibrinolytic activity on both fish and fish plus fish oil.	The authors review other reports on fibrinolytic activity and note the inconsistency in findings.
Brown and Wahle, (Ref. 184).	Crossover trial of 15 g fish oil (MaxEPA, 4.5 g/day EPA plus DHA) with or without 400 IU vitamin E for 4 weeks each with 2-week washout between.	11 healthy females.	Thiobarbituric acid reactive substances, total tocopherol by fluorometric assay, whole blood aggregation by electrical impedance after collagen stimulus.	NS conjugated dienes, creatine kinase, or TBARS in the platelet poor plasma; ↑ total plasma TBARS on both, but less with added vitamin E; ↑ glucose on fish oil without vitamin E.	Small number of subjects; considerable variance in many measures with the exception of plasma TBARS. The interaction between glucose suggests a mechanism to address potential adverse effect in diabetics.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Clarke et al. (Ref. 189).	Noncontrolled supplementation, 1 g fish oil/day (0.3 g EPA plus DHA, MaxEPA) increasing in 1 g increments monthly to 5 g/day for months 5 and 6.	7 male and 4 female adolescents with FHL type II (5 type Iia, 6 type Iib).	Usual low cholesterol, low saturated fat diets, 2 received colstipol.	NS TG's cholesterol, LDL, HDL. Increased nose bleeds during fish oil treatment versus before and after.	Numbers of observation at each time per subjects not given for blood lipids measurements, so fish oil amounts for "after" treatment not clear. Platelet count and other biochemistries described as normal, but no data of effects if fish oil are described.
Cobiac et al. (Ref. 190).	Randomized double-blind placebo-controlled, 2 week run-in on restricted Na intake plus 8 to 1 g sunflower oil capsules and 8 to 600 mg slow release NaCl. Then 4 weeks on fish oil (8 g HIMEGA, 4.2 g EPA plus DHA) on either lo or normal Na, and crossover to alternate Na for 4 weeks.	50 Elderly, healthy subjects.	Blood pressure in sitting position.	↓ sys, dias blood pressure on Na restricted diet plus fish oil; NS on fish oil only; NS on sunflower oil (the run-in treatment).	Note that sodium restriction alone had no effect. 55 elderly subjects started the experiment but only 50 completed the study. No explanation was given why some subjects were dropped out. Dietary intake and compliance were not controlled. No fatty acid analysis of the diet and/or the control and test oils. No washout period in between cross-over.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Davidson et al. (Ref. 191).	Three experiments: 1. Dose response study with fish oil, (7.2, 5.4, 3.6 g EPA plus DHA/day, SuperEPA) for 6 weeks sequentially, 6-week washout between doses; highest dose crossover to olive oil 2. Crossover of MaxEPA at 4.8 g EPA plus DHA/day each, 6 weeks, 6-week washout 3. Uncontrolled supplementation of cases from a 4 year period, 148 subjects treated for 6 week periods with various fish oil.	1. 16 Type II-B hyperlipidemic patients. 2. 12 Hyper triglyceridemic type IV. 3. 148 Patients of different hyperlipidemias.	1. Stable AHA phase 1 diet for 3 months prior to and during study. 3. Stable on AHA phase I or Phase II dietas.	1. ↓ TG, cholesterol on 7.2, 5.6 g EPA plus DHA/day, NS cholesterol on 3.6 g EPA plus DHA/day; NS HDL; cholesterol ↑ on olive oil. 2. SuperEPA ↓ cholesterol more than MaxEPA; MaxEPA ↑ LDL, HDL, HDL, versus SuperEPA; NS HDL. 3. Each MaxEPA, SuperEPA, Promega ↓ TG's, cholesterol; ↑ HDL in Type Iib on SuperEPA only; ↓ LDL in familial hypercholesterolemia after SuperEPA, NS Lp(a).	Olive oil control increased TG cholesterol versus run-in diet. Design doesn't control for polyunsaturated fatty acids. Larger decrease in cholesterol by SuperEPA may be due to its reduced content of saturated fatty acids. Capsule counts were used to assess compliance.
Eritsland et al. (Ref. 195).	Randomized to aspirin (300 mg/g) for 1 week, followed by 4 week on fish oil (Norak, 85 percent ethyl esters of EPA plus DHA, 3.4 g/day) or 4 week on fish oil followed by 1 week on aspirin.	22 female patients with stable CHD.	4 week run-in. Beta blockers used by 9 patients, nitrates allowed. Usual diet.	↓ TG's by fish oil; ↓ cholesterol by fish oil plus aspirin; ↑ HDL in fish oil only.	2 minor bleeding episodes on aspirin, none on fish oil. No was out between treatments, small number of subjects makes differences in response to fish oil only and fish oil plus aspirin questionable.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Fahrer et al. (Ref. 196).	Self selected to treatment of fish (1.5 to 2.0 g EPA plus DHA/day), fish oil (Sanomega s18, 3.1 g EPA plus DHA/day), normal diet for 2 months.	21 female, 21 male healthy volunteers.	No run-in, fish was pink salmon, tuna, herring, mackerel, pilchard. Fish consumption recorded in daily diary.	↓ TG's in both fish and fish oil groups; NS cholesterol, HDL; decrease in TG correlated with increase in EPA.	Baseline blood lipid values were comparable in the self selected groups.
Ferretti et al. (Ref. 197).	Nonblinded, longitudinal design, 10 week run-in on placebo (15 g mixed fat); 10 weeks with 15 g fish oil (anchovy oil, 6.5 g EPA plus DHA); 8 weeks of 15 fish oil plus 200 mg vitamin E.	40 nonsmoking females.	PGE-M by a stable isotope dilution method developed in the authors' laboratories.	Fish oil alone and fish oil supplemented with vitamin E produced comparable results on average, with the mean values μg PGE-M excreted per 24 hours in control, fish oil and fish oil plus vitamin E of 15.41 ± 2.12 , 12.51 ± 1.78 and 12.77 ± 1.85 , respectively. Paired t-test showed a significant ($p < 0.002$) reduction between baseline and fish oil treatment.	PGE-M is the sum of PGE ₁ plus PGE ₂ , derived from AA. The EPA-derived PGE ₃ was not measured. Dietary intake was well controlled. Prolonged use of fish oil supplementation was not recommended. The range of baseline values was from 3.8 to 60.9 $\mu\text{g}/24$ hours. There was no apparent relationship between initial values and magnitude of the change, and there were many individuals who had substantial differences for the two fish oil treatments. Thus, the significance of an average change of about 20 percent is not clear.
Force et al (Ref. 200).	12 g fish oil (n=8) (6 g EPA plus DHA, Promegre) or 16 g fish oil (n=6) (8 g EPA plus DEA), 6 weeks. After 6 weeks on fish oil only, concurrent increasing daily dosages of ASA (50, 100, 225, 1,300 mg) 2 weeks each.	14 females, 2 males, clinically stable but advanced CHD.	Gas chromatography-mass spectroscopy	Fish oil ↓ TXA ₂ , 38 percent, with ASA ↓ 97 percent at each dose; fish oil and ASA each ↓ PGI ₂ , (ASA more than fish oil); fish oil ↑ PGI ₂ , but ASA did not increase PGI ₂ .	This is a study on the mechanism of action of fish oil. No concurrent placebo control. Dietary intake was not controlled.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Franceschini et al. (Ref. 202).	Uncontrolled supplementation study of 6 g/day fish oil (Norsk hydro, 2.8 g EPA plus 1.7 g DHA), 6 weeks.	5 healthy subjects.	HDL subfractions assayed by nondenaturing polyacrylamide gel electrophoresis.	NS cholesterol, HDL, ↑ HDL ₂ , ↓ HDL ₃ , ↑ HDL ₂ /HDL ₃ mass ratio.	Dietary intake was not controlled. Compliance was checked by plasma PL fatty acid composition. Small study.
Gans et al. (Ref. 203).	Randomized double-blind study (fish oil, source not specified, 1.8 g EPA plus 1.2 g DHA/day) versus corn oil, 4 months.	Stable claudication patients; 37 enrolled, 16 per group completed.	Supine blood pressure at rest and 1, 6, 10 min post exercise. Fibrinogen by commercial kit.	↓ Diastolic by both groups, ↓ systolic only in CO group; ↑ RBC deformability; NS cholesterol, LDL; fibrinogen; ↑ HDL, HDL ₂ ; ↓ TG's; NS pain, walking distance.	Wide variations of age (18 to 80 years), Dietary intake was not controlled. Compliance was checked by plasma PL fatty acid composition. Blood pressure was lowered in both groups.
Gazso et al. (Ref. 104).	Randomized double-blind placebo-controlled Efamol-marine (1.2 g EPA) versus Efamol (v olive oil) 12 capsules/day 6 weeks.	17 normal healthy 6 males, 11 females 6-Efamol marine, 5-Efamol, 6-olive oil.	ic conversion, MDA, platelet aggregation to ADP.	↓ Platelet aggregation by Efamol-marine; ↓ MDA in all groups.	Groups differ in the endpoints at beginning of the experiment, so it is difficult to interpret changes. The MDA ↓ may be due to the vitamin E added.
Gerhard et al. (Ref. 206).	3-period crossover of three fish diets; Dover sole (2 g EPA plus DHA), Salmon (4 g EPA plus DHA), or sablefish (3.4 g EPA plus DHA), 18 d each with 3-week washout between.	21 normo-triglyceridemic females.	ApoB standardized to Centers for Disease Control standards. VLDL and LDL precipitated with magnesium phosphotungstate, HDL enzymatically in the supernatant.	Salmon, sablefish diets ↑ cholesterol, apoB, LDL. Sole ↓ HDL, ↑ LDL; Sablefish ↓ HDL.	Diets were comparable for total fat, saturated fat. Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.
Goren et al. (Ref. 207).	Uncontrolled supplementation study, 150 to 200 mg/kg (EPAGIS) (0.86 to 2.3 g EPA plus DHA/day) 8 weeks.	16 Patients with end stage renal disease, 6 type Iib, 1 type Iia, 8 type IV.	Blood lipids before and after supplementation. cholesterol by microenzymatic method. Apoproteins by turbidity assays.	↓ TG; NS cholesterol, ↓ cholesterol/HDL in a subset of excessively hyperlipidemic subjects; NS blood pressure, platelet counts, apoA ₁ :apoB.	Fish oil dosage was adjusted to the body weight of chronic renal failure young patients (7 to 18 years).

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Green et al. (Ref. 209).	Randomized double-blind placebo-controlled crossover 15 g fish oil (EPAGIS, 4.3 g EPA plus DHA) versus corn:olive oil mix, 8 weeks each treatment with 4 week wash out.	27 Hyperlipidemic subjects, 15 type Iib, 12 type IV.	Cholesterol, HDL, TG by commercial kits. Apoprotein by immunoturbidity assay.	Overall NS cholesterol, LDL. Type Iib: ↓ TG, cholesterol, LDL, HDL. Type IV: ↓ TG; NS cholesterol, LDL, HDL. Both: NS platelet count, platelet aggregation, RBC deformability, apoB, apoA,; ↓ blood viscosity.	Substantial amount of other omega-3 fatty acids in this supplement (0.50 g 18:3, 0.45 g 18:4 and 0.42 g 22:5) per day. Patients were on weight-maintenance diet but no cal percent or wt percent of each component was given. Compliance was shown on blood lipid analysis.
Haglund et al. (Ref. 210).	Double-blind crossover study 30 mL/day of low vitamin E fish oil (9.6 g EPA plus DHA/day, Eskimo-3 or Inuit-3) or same oil supplemented with 1.5 IU vitamin E, 3 week.	12 Normal healthy subjects.	Lipids, glucose, Lp(a), fibrinogen.	Both fish oil ↓ TG's; ↑ HDL, glucose; NS cholesterol, Lp(a), aopB, insulin. Low vitamin E fish oil ↑ MDA, fructosamine; ↓ vitamin E. High vitamin E fish oil ↓ TG's, fibrinogen.	High vitamin E produced some effects independently of the fish oil, underscoring the need the control for polyunsaturated fatty acids, and have adequate vitamin E in the test substances.
Hamazaki et al. (Ref. 215).	Uncontrolled supplementation study, 1.8 g EPA ethyl ester/day, (laboratory purified), 6 months.	16 Diabetics, 5 IDDM, 11 NIDDM.	Albumin by radioimmunoassay with a commercial kit. HbA _{1c} by high performance liquid chromatography.	NS TG, cholesterol, glucose, HbA _{1c} , systolic diabolistic blood pressure, blood viscosity; ↓ albumin excretion, hematocrit.	NS body weight. Compliance was checked by blood EPA level. Dietary intake was not controlled. In IDDM fasting glucose was reduced, and barely missed statistically significance. Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.
Hansen et al. (Ref. 216).	Crossover trial of 25 mL cod liver oil/day (6.25 g EPA plus DHA), 8 weeks versus no supplement.	20 healthy female and 20 healthy male subjects.	Whole blood clot (produced by added thrombin) lysis time to complete lysis. t-PA by commercial ELISA kit. Fibrinogen by spectrophotometric assay.	NS fibrinogen, fibrinolytic activity, t-Pa; ↓ TXB ₂ .	Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.

TABLE 2-CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Harats et al. (Ref. 217).	Parallel untreated controls versus 10 g MasEPA/day (3 f EPA plus DHA), 4 weeks.	a. Smokers 6 MaxEPA, 5 controls. b. Smokers 3 control, 4 fish oil only, 4 fish oil plus 400 mg smokers. c. Nonsmokers.	40 hour smoking abstinence and overnight fast prior to blood draw; 90 minutes 4 to 6 cigarettes smoked, for second blood draw.	↓ TG; NS cholesterol, HDL; fish oil ↑ TBARS in plasma presmoking ↑ TBARS after smoking.	TBARS in plasma, and LDL, more responsive to cigarettes than fish oils. Most, but not all of the increase due to fish oil alone could be blocked by the added vitamin E.
Harris et al. (Ref. 218).	Uncontrolled supplementation trial of fish oils plus aspirin; 325, 80, 80 mg aspirin for 3 days; 4 day wash out; 2 weeks on 4.5 g EPA plus DHA (SuperEPA); 325, 80 80 mg aspirin plus SuperEPA for 3 days.	8 healthy males.	Bleeding times by Simplate II. Platelet aggregation to AA, collagen, PAF and AA in combination with the other agonists.	Bleeding NS on fish oil; fish oil plus aspirin same as aspirin only; fish oil and fish oil plus aspirin NS on platelet sensitivity to AA, collagen, PAF, but fish oil ↓ extent of aggregation to collagen.	Medications were controlled but diet was not controlled. Short-term study with a small number of subjects may explain inconsistencies with other comparable studies. The study may not have adequate statistical power to determine whether bleeding time increases of aspirin and fish oil are additive or greater than additive.
Harris and Windsor (Ref. 220).	Uncontrolled supplementation study on postprandial lipemia with of fish oil (2.2 g EPA plus DHA, Dale Alexander Omega-3), random assignment or emulsion for 4 weeks.	12 male and 4 female healthy normolipidemic subjects.	Bleeding times by Simplate. Background diets had 32 to 36 percent fat with 12 to 14 percent as saturated, 12 to 13 percent as monounsaturated, and 6 percent as polyunsaturated. Test meal provided 1 g fat/kg (61 percent of total calories: 32 percent of calories from saturated fatty acids; 13 percent from monounsaturated fatty acids, 7 percent from omega-3 fatty acids. Two hour blood samples through 10 hours post meal.	↓ TG's VLDL; NS cholesterol, LDL, apoB, apoA1, HDL3, vitamin E, Lp(a); ↑ HDL, HDL2. ↑ Bleeding time; NS RBC deformability. Postprandial lipemia reduced about 40 percent.	No medications. No difference between capsules and emulsion in test meal, possibly because most fat was from other diet components.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Harris et al. (Ref. 219).	Randomized dose-response, 1.25 to 5 g EPA plus DHA/day (Promega) 6 months.	28 Hyperlipidemic patients.	Blood lipids, Simplate II for bleeding times.	↓ TG in dose-related manner 1 month and 6 months, except lowest dose NS at 6 months, ↓ VLDL on all but lowest dose; NS cholesterol, LDL, HDL, HDL ₂ , except 2.5 g/day at 6 months ↑ LDL, HDL). ↑ Bleeding times on 2.5, 5 g/g RBC deformability largely unaffected.	Discrepancies among studies, methodologies were discussed. Irregularities may be due in part to small sample size. 4-week washout returned most values to pretreatment levels.
Hendra et al. (Ref. 222).	Randomized, double-blind, placebo-controlled trial of 10 g/day MaxEPA (3 g EPA plus DHA) versus olive oil, 6 weeks.	80 Noninsulin-dependent diabetic subjects.	Fibrinogen by Clauss, HDL by precipitation, LDL by calculation.	Transient ↑ glucose; NS HbA _{1c} . ↓ TG; NS cholesterol, HDL; ↑ LDL (versus baseline). ↓ Spontaneous platelet aggregation, but NS response to induced aggregation. ↓ blood pressure in both treatments.	Large, carefully controlled study in an at-risk population. Olive oil control doesn't allow conclusions about omega-3 fatty acid-specific effects.
Homma et al. (Ref. 224).	Uncontrolled supplementation test of 2.7 g/day purified EPA ethyl ester (source not specified), 12 weeks.	15 outpatients.	Ad libitum diets. Blood samples after 12 hour fast. Plasma lipids by ultracentrifugation every 4 weeks.	↓ cholesterol, TG, apoB, small dense LDL; ↑ large light LDL, lipid transfer protein activity; NS HDL, HDL, HDL ₃ , apoA ₁ , apoC, apoE.	Authors state the relative atherogenicity of large light LDL and small dense LDL is controversial.
Jensen et al. (Ref. 226).	Sequential dose-response with 1, 3, 6 g EPA plus DHA (Shaklee EPA), 4 weeks each with 3-week washout between.	14 healthy males and 4 healthy females.	1 month run-in on fish free diet, otherwise diet not controlled. Bleeding times by Simplate II. Lipids by auto laboratory method.	↓ TG; VLDL on 6 g dose; ↑ HDL and HDL/LDL ratio on 6 g dose, but baseline HDL changed; NS cholesterol, LDL.	Changes in baseline HDL not shown.
Kremer et al. (Ref. 228).	Randomized double-blind placebo (olive oil) controlled trial of 3.25 or 6.5 g EPA plus DHA/day (ethyl esters, Pharmacs), 24 weeks.	49 with rheumatoid arthritis completed this study.	IL-1 by bioassay.	↓ IL-1 38 percent 41 percent and 55 percent in olive oil, low and high fish oil groups, respectively; NS IL-2 in both fish oil groups.	Actual doses were adjusted per kg body weight.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Li and Steiner (Ref. 234).	Dose-response, parallel design: 4.8, 9.6, or 14.4 g EPA plus DHA/day, (source not specified), 3 weeks.	5 Normal healthy subjects each dose.	Platelet adhesion measured ex vivo in laminal flow chamber, using purified substrates.	↓ Platelet adhesion to collagen I and fibrinogen, near maximal response at 3 g EPA/day; speen of return to baseline values in the washout was directly related to dose.	This procedure reduces formation of thrombi, dilutes platelet derived factors. Measures direct interaction of platelets with surface matrix. Study design doesn't allow conslusions about omega-3 fatty acid-specific effects.
Lindgren et al. (Ref. 235).	Metabolic ward crossover of salmon versus prudent diet (30 percent fat). 20 day run-in, 40 days each diet. Salmon diet provided 2.1 percent of calories as omega-3 fatty acids, (approximately 5 g/day EPA plus DHA).	9 normolipidemic females.	Plasma proteins measured by competitive ELISA except apoA, by radioimmunoassay.	NS cholesterol, LDL, HDL _{2a} , HDL _{3a} , apoB, apoE, Lp(a); ↓ TG, HDL _{3a} , HDL _{3b} , apoA ₁ , apoA ₂ ; ↑ HDL, HDL _{2b} .	Carefully designed metabolic ward study, using practical level of omega-3 fatty acids, and Fat (saturated fatty acids and omega-6 polyunsaturated fatty acids) carefully controlled. Details on effects of lipoprotein subfractions. Two assay methods for Lp(a) gave same result.
Mallo et al. (Ref. 241).	Uncontrolled supplementation with fish oil, (6.7 g EPA plus DHA/day, EPAX-5000), 6 weeks and 4-week washout.	Normolipemic subjects with very high (?) and very low i.e., undetectable Lp(a) levels (7).	4 week run-in. normal diets. Platelet aggregation to collagen, thrombin.	↓ TG, platelet aggregation; NS cholesterol, LDL, HDL, Lp(a).	Comparable lipid and platelet responses for the low Lp(a) and high Lp(a) groups.
Marckmann et al. (Ref. 244).	observational, sequential diets of fish (3.4 g/day EPA plus DHA, 10 d) uncontrolled (18 d), and meat (10 d).	12 healthy females.	Clause fibrinogen assay, t-PA and PAI-1 antigens by ELISA.	NS cholesterol, HDL, TG, fibrinogen; TG ↓ on both diets; ↓ PAI-1 and t-PA antigen, PAI activity and ↑ t-PA activity on meat, but NS on fish.	Since both diets produced changes with respect to the initial diets (that were uncontrolled) it is difficult to ascribe any change to the omega-3 fatty acids. However, the changes on the meat diet are more in line with reduced CHD risk than the lack of change on the fish diet.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Meland et al. (Ref. 247).	Double-blind, randomized multi-center placebo (corn oil- olive oil mix) controlled trial, 20 mL MaxEPA/day (6.8 g EPA plus DHA), 6 weeks.	40 females mild hypertension.	Calibrated instruments at 8 centers. Time of day for measurements was controlled.	NS blood pressure, cholesterol; ↓ TG's on fish oil; ↓ cholesterol/HDL ratio in both groups.	Power to detect a 5 mm blood pressure difference was 96 percent; a 10 percent cholesterol difference was 61 percent. Cholesterol/HDL ratio decrease in placebo was nearly more than that after fish oil ($p < 0.07$). 11 of 14 subjects on fish oil guessed their assignment correctly.
Meydani et al. (Ref. 248).	Uncontrolled supplementation study 2.4 g/day EPA plus DHA (Promega), 3 months.	25 males.	Blood at 1, 2, 3 months.	↓ TG's; ↑ lipid peroxides.	6 IU vitamin E may not be adequate.
Molvig et al. (Ref. 250).	Randomized, double-blind Placebo-controlled trial of 1.6, 3.2 g EPA plus DHA (Pikaso) versus fatty acid blend, 7 weeks.	25 Healthy subjects and 8 IDDM subjects.	Isolated monocyte cell cultures. TNF and IL-1 by commercial ELISA kits.	↓ IL-1B immunoreactivity on high dose only; NS after low dose. NS TNF- α ; ↓ proliferative response.	Placebo had 20 percent polyunsaturated, 38 percent monounsaturated fatty acids. Spontaneous and LPS-stimulated leucotriene B, and PGE, secretion did not differ among groups at baseline or after 7 weeks of treatment. IL-1 returned to baseline with 3-week washout.
Mori et al. (Ref. 251).	Matched (age, weight) and randomized to 15 g MaxEPA/day (4.5 g EPA plus DHA) or olive oil, 4 weeks.	32 females with peripheral vascular disease.	No aspirin for 14 days prestudy platelet aggregation to PAF, collagen.	↑ cholesterol, LDL, HDL ₂ ; ↓ TG by fish oil, (but olive oil ↓ cholesterol, LDL); ↓ platelet aggregation by fish oil, but olive oil ↑ aggregation.	Compliance by capsule count. Changes in control make interpretation difficult. Olive oil does not control for polyunsaturated fatty acids.
Mori et al. (Ref. 252).	Matched groups randomly assigned to 15 g/day MaxEPA (4.5 g EPA plus DHA), olive oil, or olive oil plus cholesterol.	27 normolipidemic insulin-dependent female diabetics.	HDL by heparin, manganese chloride precipitation, followed by separate precipitation of subfractions. LDL by calculation.	NS cholesterol, LDL, HDL; ↑ HDL ₂ , ↓ HDL ₃ , TG.	Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Mueller et al. (Ref. 253).	Randomized double-blind crossover trial of 8 g EPA plus DHA (Promega) versus olive oil, 21 d.	12 Healthy adults.	Bleeding times by Simplate II before and after administration of 325 mg aspirin. Excludes subjects with platelet or coagulation disorders, thrombocytopenia, ethanol.	Fish oil-NS bleeding time versus baseline but ↑ versus olive oil both before ($p < 0.02$) and after (NS) aspirin. ↓ TG on fish oil, platelet count, WBC count; NS cholesterol, LDL, HDL.	Trend toward ↑ HDL versus baseline, but olive oil in same direction, some order effects confound results.
Muller et al. (Ref. 253a).	Multicenter observational trial of 135 g canned mackerel paste (4.7 g/day EPA plus DHA) or meat paste.	84 healthy females.	Published methods for factor X, antiplasmin, plasminogen. Fibrinogen by Clause.	NS fibrinogen, other blood coagulation measures (only ↑ factor X), or fibrinolysis measures; meat ↓ plasminogen.	Compliance by lithium excretion. Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.
Mullertz et al. (Ref. 254).	Uncontrolled supplementation, 0.55 g EPA plus DHA/day (Pikazol), 21 days.	7 Healthy adults.	Normal diets, PAI-1 and u-PA by ELISA kits.	↓ α-Tocopherol; NS cholesterol, TG's; ↑ PAI-1; NS t-PA, u-PA.	Suggests that differences reported for PAI-1 are due to the assay used, with the double antibody assay used in this study, and the monoclonal antibody used by Emeis et al. providing specificity. Concludes that fish oil decreases fibrinolytic activity.
Nelson et al. (Ref. 256).	Metabolic ward crossover of salmon, prudent diet (30 percent fat). 20 day run-in, 40 days each diet. Salmon diet gave 2.1 percent of calories as omega-3 fatty acids, (approximately 5 g/day EPA plus DHA).	9 normolipidemic females.	Platelet aggregation to ADF, AA, collagen, thrombin: threshold and maximum response. Bleeding time by Simplate II.	NS bleeding time; salmon diet ↓ platelet counts NS platelet response to collagen, thrombin but ↓ sensitivity to ADP.	Carefully designed metabolic ward study, using practical level of omega-3 fatty acids, and Fat (saturated fatty acids and omega-6 polyunsaturated fatty acids) carefully controlled.

TABLE 2—CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Nikklila (Ref. 258).	Randomized, double-blind, placebo (corn oil) controlled, crossover, 2.4 g EPA plus DHA/day as ethyl ester (EPA X 6000EE, Almarin), two 4-week periods with a 4-week washout between, followed by open study of 3.6 g EPA plus DHA/day for 4 weeks.	32 females with CHD, increased TG and decreased HDL, 62 percent were overweight.	Lipid lowering diet for 4 weeks prior to study.	NS cholesterol, LDL, HDL, HDL/cholesterol ratio, apoA ₁ , apoB; ↓ TG's; During open phase those with severe hypertriglyceridemia had ↑ HDL/cholesterol.	HDL inversely related to TG in study group pretreatment.
Nye et al. (Ref. 259).	Randomized, fish oil and its placebo were double-blind 1. Aspirin 300 mg plus dipyridamole 75 mg. 2. 3.6 g EPA plus DHA (MaxEPA). 3. Olive oil, up to 1 year.	79 females, 29 males post PTCA referred for angina, none had grafts.	Angiography (blind) at one year or before in those with anginal symptoms; restenosis defined as a loss of 50 percent or more of the gain produced by PTCA.	NS angina (trend toward less in A/D and fish oil groups D restenosis by EPA (11 percent versus 30 percent for olive oil) MaxEPA not different in any regard versus A/D NS in any blood lipids in a subset (n= 42).	No deaths in any group through 1 year, 93 percent follow-up rate. Results suggest that MaxEPA is as useful or more so than aspirin/dipyridamole.
Oh et al. (Ref. 261).	Randomized crossover of 4 normal eggs versus 4 omega-3 fatty acid-enriched eggs/day (4.5 g EPA plus DHA/day), 2 week run-in, 4 weeks each treatment.	9 female and 3 male healthy volunteers.	Recumbent blood pressure; VLDL by ultracentrifugation, HDL by manganese-heparin precipitation.	Omega-3 fatty acid-enriched eggs did not ↑ cholesterol, but regular eggs did. Omega-3 fatty acid-enriched eggs ↓ TG in one group.	One of the groups used butter to prepare eggs, changing the P:S ratio. Pooled data were not given despite absence of order effects for most variables. ↓ LDL in one group; NS HDL in either treatment. Systolic blood pressure ↓ in both groups, diastolic only in one.
Olivieri et al. (Ref. 263).	Uncontrolled supplementation trial of 20 mL fish oil/day (3.0 g EPA plus DHA, source not specified), 8 weeks.	20 hyperlipidemic 16 female, 4 male.	No hypolipidemic drugs for 15 days pre trial. blood pressure by blinded nurse.	↓ Systolic diastolic blood pressure, TG; NS cholesterol, HDL, vitamin E; ↑ glutathione peroxidase activity in RBCs and platelets, ↓ MDA.	Design doesn't allow conclusions about omega-3 fatty acid-specific effects.

TABLE 2—CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Owens and Cave, (Ref. 264).	Observational study, 15 g/day MaxEPA (4.5 g EPA plus DHA) 4 weeks.	6 normal females.	Simplate II for bleeding time. Platelet adhesion in Baumgartner chamber using everted rabbit aorta. Prothrombin time, WBC and platelet count by automated methods.	NS WBC, prothrombin time, platelet adhesion, bleeding time.	Trend toward increased adhesion with duration of feeding. Assay method measures platelet changes, but does not assay vessel wall changes. Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.
Rapp et al. (Ref. 268).	Uncontrolled supplementation study of MaxEPA at 6 percent of calories (16 to 21.3 g EPA plus DHA/day), 6 to 120 days.	11 patients, 9 female, 2 male with obstructive atherosclerosis scheduled for peripheral vascular surgery.	Excludes subjects with habitual fish intake. 15 endarterectomy specimens. Control specimens from 18 nonfish consuming subjects undergoing vascular reconstruction.	Fish oil increased content of omega-3 fatty acids in atherosclerotic lesion linearly with duration of feeding, although plasma enrichment of omega-3 fatty acids plateaued by 2 to 3 weeks; ↓ cholesterol; NS TG's, platelet counts, bleeding times.	Shows incorporation of omega-3 fatty acids into plaque, especially DHA. Relevance to CHD not known. Not a specific effect of omega-3 fatty acids, but would be expected to polyunsaturated fatty acids. High amount of omega-3 fatty acids.
Saynor and Gillot (Ref. 276).	Uncontrolled long-term supplementation with 20 mL/day MaxEPA during year 1, 10 mL/day thereafter.	365 During 1 month to 40 at 84 months. 47 percent had survived a heart attack, 49 percent had angina.	Total cholesterol by enzymatic assay. HDL after precipitation.	↓ TG; ↓ cholesterol only for initial high cholesterol; ↑ HDL for total group; NS LDL; ↓ fibrinogen.	Large attrition makes it difficult to ascribe changes to fish oil (responders to treatment are more likely to stay in the study). Lack of blinding also may have contributed to bias. Some data were presented for all subjects only, other data only for subsets. Estimates of deviation from mean values not shown. Lack of control prevents conclusions regarding effects to omega-3 fatty acids.
Schmidt et al. (Ref. 277).	Dose-response study 1.3, 4, 9 g EPA plus DHA/day (Pikaso), 3 periods of 6 weeks/amount. Randomized to increasing or decreasing dose. 6-week washout.	10 healthy females.	Simplate II for bleeding times; t-PA, PAI by commercial kits; fibrinogen by thrombin clotting time.	NS cholesterol, LDL, platelet aggregation; ↑ HDL, bleeding time on 4 and 9 g/day, PAI and t-PA antigen after 9 g/day; ↓ TG, fibrinogen.	Design doesn't allow conclusions about omega-3 fatty acid-specific effects.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Schmidt et al. (Ref. 270).	Uncontrolled supplementation with 4 g EPA plus DHA/day (Pikasol), 6 weeks.	10 Untreated hypertensives.	Supine blood pressure.	NS cholesterol, LDL, HDL, TG, platelet aggregation to collagen, ADP, systolic, diastolic blood pressure, bleeding time; ↓ cholesterol/HDL ratio, fibrinogen, monocyte chemotaxis.	Design doesn't allow conclusions about omega-3 fatty acid-specific effects. Absence of significant change in plasma TG despite 25 percent decrease suggests inadequate sample size. Before and after compared by Pratt's test.
Schmidt et al. (Ref. 279).	Uncontrolled supplementation with 1.3 to 9.0 g EPA plus DHA/day (Pikasol, MaxEPA or cod liver oil), most for 6 weeks, angina subjects for 12 weeks.	Various at-risk subjects with angina (14), IDDM (10), hyperlipidemia (17), hypertension (10), and healthy subjects (46).	Normal diets. Lp(a) by two-site immunoradiometric test kit.	NS Lp(a) in any group.	Reports Lp(a) data for subjects from 5 previous Schmidt reports (Refs. 133 through 135), and the current refs above. Design doesn't allow conclusions about omega-3 fatty acid-specific effects.
Shapiro et al. (Ref. 203).	Uncontrolled supplementation with 10 g MaxEPA/day (5.4 g EPA plus DHA/day), 6 weeks, 10-week washout.	10 normolipidemic healthy females.	3 Samples per time point, 2 to 3 days apart.	↑ cholesterol, LDL, HDL, vitamin E, retinol versus presupplementation and washout; ↓ TG versus washout.	Multiple samples per treatment reduces day-to-day fluctuations, magnitude of changes: Cholesterol 6 percent; LDL 9 percent; HDL 11 percent versus average of pretreatment and washout values.
Singer et al. (Ref. 205).	Randomized to propranolol (P), or fish oil (2.9 g EPA plus DHA/day, source not specified), for 36 weeks, or (P) only (12 weeks) then P plus fish oil (12 weeks) then P plus olive oil placebo (12 weeks). Each followed by 4 week washout.	47 female patients with mild essential hypertension.	Two baseline blood pressure measure 4 weeks apart, blood pressure measured in triplicate at fixed time and post 2 hours of rest each 12 weeks.	P ↓ systolic, diastolic blood pressure, recumbent and upright; fish oil ↓ systolic, diastolic blood pressure in recumbent and upright; Some additive effects of P plus fish oil.	Olive oil control doesn't control for polyunsaturated fatty acids. Study duration and multiple measure (each 12 weeks) shows blood pressure lowering effect is persistent.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Sirtori et al. (Ref. 286).	Randomized, three-arm crossover of 6 g fish oil (Norsk Hydro, 4.5 g EPA plus DHA ethyl esters) versus olive oil (middle arm for each sequence) versus corn oil for 6 weeks each. 1 month run-in and 4 week wash-out between each arm with low saturated fat diet.	12 Type Iia hyperlipidemics.	Lipids by enzymatic assays, apoproteins by immunoturbidity assay. Platelet aggregation versus AA. TXB ₂ by radioimmunoassay. Superoxide by spectrophotometry.	Fish oil ↓ cholesterol, LDL, ↑ HDL; Olive oil ↓ LDL, ↑ HDL; Corn oil ↓ HDL, apoB. Platelet aggregation ↓ by all three oils. Fish oil ↓ superoxide in monocytes.	Excellent design. Divergent results from another recent study with comparable design (Bona et al. (Ref. 178) that used the same amount and form of fish oil supplement and same control (corn oil), suggesting that responses to supplemental oils may be different for different sub-populations. Absence of change in platelet aggregation may also be a population specific finding.
Spannagl et al. (Ref. 287).	Uncontrolled supplementation with 8.1 g EPA plus DHA/day (PGE-technology), 4 weeks.	13 (3 male 10 female) near normoglycemic type I diabetics.	Fibrinogen by turbidity assay. t-PA, PAI by test kits.	NS clotting tests, t-PA; ↑ PAI, fibrinogen; ↓ TG.	Design doesn't allow conclusions about omega-3 fatty acid-specific effects. Fairly high amount of omega-3 fatty acids in this nonnormal population.
Trial of Hypertension Prevention Collaborative Research Group (Ref. 289).	Randomized life style interventions and double-blind, placebo-controlled nutritional supplement interventions including 3.0 g omega-3 fatty acids/day (source not specified), 6 months.	2182 female and male with diastolic blood pressure 80 to 89 mm Hg.	Sitting blood pressure after 5 minute rest. Measurements for baseline, 3 and 6 months were made in triplicate on 3 different days. On the fish oil arm there were 161 active and 157 control subjects.	NS systolic, diastolic blood pressure.	Large, multicenter design with many internal comparisons.
Vandongen et al. (Ref. 291).	2 week run-in, observational trial of 15 g/day MaxEPA (4.5 g EPA plus DHA) versus no supplement.	22 female insulin-dependent diabetics.	Double precipitations for HDL subfractions.	↑ cholesterol, LDL, HDL, HDL ₂ ; ↓ TG, HDL ₃ .	Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Virella et al. (Ref. 295).	Double-blind placebo (olive oil) controlled study of 2.4 g EPA (DHA not given) 6 weeks.	6 normal subjects.	Blood samples at baseline, 3, 6, 14, 20, 28. Humoral immune response.	↓ TG in 3/4 on fish oil; variable in vivo response to tetanus toxoid booster; ↓ in vitro response to tetanus toxoid, ↓ IL-2 release.	Small number of subjects limits conclusions. Increase in bleeding reported in 2/4.
Vohwinkel et al. (Ref. 296).	Randomized, double-blind crossover of 6 g EPA plus DHA/day (source not specified) versus olive oil, 3 weeks.	48 Healthy subjects.	Glucose tolerance tests to 100 g oligosaccharides.	↑ Fasting glucose, insulin at 4 hours post load. Response of glucose to load affected differently by fish oil, depending on initial insulin response; among low responders fish oil increased insulin response and decreased glucose; among high insulin responders, fish oil reduced insulin response and lowered glucose response.	Complex results according to insulin responsiveness. Olive oil doesn't control for effects of polyunsaturated fatty acids.
Wander and Patton, (Ref. 297).	3-period crossover of three fish diets; Dover sole (2 g EPA plus DHA), Salmon (4 g EPA plus DHA), or sablefish (3.4 g EPA plus DHA), 18 day each with 3-week washout between.	23 normo-triglyceridemic females.	Bleeding time by Simplate. Platelet aggregation versus collagen, ADP, TXB ₂ by radioimmunoassay.	Salmon ↑ bleeding time. Sablefish ↓ platelet aggregation to collagen; Both sablefish and salmon ↓ aggregation to ADP, ↓ TXB ₂ .	Diets were comparable for total fat, saturated fat. Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.
Weintraub et al. (Ref. 298).	Metabolic ward study. Crossover to 3 isocaloric diets: saturated fat; omega-6 fatty acids and omega-3 fatty acids (3.4 g/day EPA plus DHA), 25 days each with 5 to 7 day wash-out.	8 normolipidemic females.	Vitamin A fat load test in fasted subjects; HDL by precipitation, LDL by calculation. Lipolysis assay using human milk lipoprotein lipase.	Omega-3 diet ↓ cholesterol, TG, LDL, HDL, platelet count versus saturated fat. Omega-6 diet ↓ cholesterol, TG, LDL. NS fasting glucose, postprandial insulin. Both omega-3 and omega-6 reduced postprandial lipemia versus saturated fat. NS in lipemia between omega-6 and omega-3.	Excellent design studies both chronic and acute fat effects. Many postprandial fat effects were larger on the omega-3 than the omega-6 diets, but not statistically significant.

TABLE 2—CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Wing et al. (Ref. 299).	Double-blind, placebo (olive oil) controlled crossover trial of 15 g fish oil (Lipitac), 4.5 g EPA plus DHA, 8 weeks each.	20 Treated hypertensives maintained on blood pressure medications.	Supine and standing blood pressure. HDL after manganese chloride/heparin precipitation.	Blood pressure lower comparable on olive oil and fish oil. ↓ TG in fish oil; NS HDL on either treatment.	Study design doesn't allow conclusions about omega-3 fatty acid-specific effects.
Wojenski et al. (Ref. 300).	Sequential treatments with ethyl oleate (placebo), 6 g Res-Q1000 (3.6 g EPA plus DHA/day), or 4.0 g ethyl EPA. Washouts between phases of 5 weeks, 4 months, respectively, and 8 weeks posttreatment.	9 healthy female volunteers.	Bleeding time by Simplate II; HDL by hospital automated method, platelet aggregation to ADP, collagen. TXB ₂ by radioimmunoassay. Fibrinogen binding by (125)I-Fibrinogen versus saline.	Bleeding time ↑ on ethyl EPA; Platelet count ↓ on Res-Q1000 and ethyl EPA; Ethyl EPA ↓ cholesterol, TG, platelet aggregation; NS fibrinogen binding.	No aspirin or ibuprofen. Evidence for a greater effect by the ethyl ester than for a comparable amount of omega-3 fatty acids in a mixed TG supplement.
Wolmarans et al. (Ref. 301).	Crossover comparison of red meat to fish, (6.1 g EPA plus DHA/day) 3 week baseline, 6 week treatment, 6 week posttreatment and 3-month washout.	Healthy subjects, 12 females, 16 males.	Habitual diet.	Fish diet ↓ cholesterol, LDL, VLDL; ↑ HDL.	NS total fat but ↓ saturated fat on fish diet. EPA was 1.91 g/day versus 0.06 g/day in baseline and 0.01 g/day on meat; total omega-3 fatty acids were 6.1 g/day on fish, and 0.9 g/day otherwise.

TABLE 2--CONTINUED

Study	Study Design	Subjects	Methods	Results	Comments
Zambon et al. (Ref. 304).	Randomized crossover trial of fish oil 15 g/day SuperEPA (8 g EPA plus DHA ethyl esters), with and without glyburide 8 weeks on fish oil, 4 weeks on and 4 weeks off glyburide. Baseline treatment was glyburide alone, 4 weeks.	10 females with NIDDM.	Regular diets. Insulin by radioimmunoassay. Automated glucose analysis. Cholesterol by enzymatic methods.	Fish oil ↑ fasting glucose, NS fasting insulin, ↓ postprandial insulin. Fish oil ↑ LDL, NS cholesterol, HDL	High amount of omega-3 fatty acids may produce effects on glucose metabolism not seen with lower amounts. Effects are consistent with other reports, but absence of polyunsaturated fat control limits inferences about specificity of the effects.

Abbreviations used: apoA, apoprotein A (a protein in high-density lipoprotein) apoE, apoprotein E (a protein in many lipoproteins, most notable VLDL and HDL); ASA, acetylsalicylic acid; ATP, adenosine triphosphate; CDC, Centers for Disease Control; CHD, coronary heart disease; ELISA, enzyme-linked immunosorbant assay; MDA, malondialdehyde; NIDDM, noninsulin dependent diabetes mellitus; NS, not statistically significantly different; PGE-M, prostaglandin-M; TEARS, thiobarbituric acid reactive substances; TG's, triglycerides; TXB, thromboxane.

hydrogenated menhaden oil with an iodine number between 86 and 119 is GRAS as a direct human food ingredient with no limitation other than current good manufacturing practice. These actions complete the agency's response to a petition filed by the National Fish Meal and Oil Association.

DATES: Effective June 5, 1997. The Director of the Office of the Federal Register approves the incorporation by reference, in accordance with 5 U.S.C. 552(a) and 1 CFR part 51, of certain publications in 21 CFR 184.1472(a)(2), effective June 5, 1997.

FOR FURTHER INFORMATION CONTACT: Lawrence J. Lin, Center for Food Safety and Applied Nutrition (HFS-206), 200 C St. SW., Washington, DC 20204, 202-418-3103.

SUPPLEMENTARY INFORMATION: In accordance with 21 CFR 170.35, the National Fish Meal and Oil Association, 2000 M St. NW., suite 580, Washington, DC 20036 (current address: 1525 Wilson Blvd., suite 500, Arlington, VA 22209), submitted a petition (GRASP 6G0316) seeking affirmation that menhaden oil and partially hydrogenated menhaden oil are GRAS for use as direct human food ingredients. The petition included information about the identity of, and manufacturing processes for, menhaden oil and partially hydrogenated menhaden oil; final reports and published articles of long-term animal feeding studies with partially hydrogenated menhaden oil; information about the history of human food use of partially hydrogenated menhaden oil; and the results of an extensive search of the published scientific literature (encompassing over 2,600 articles) with respect to the safety of fish oils in general.

FDA published a notice of filing of this petition in the *Federal Register* of July 31, 1986 (51 FR 27461), and gave interested persons an opportunity to submit comments to FDA's Dockets Management Branch. FDA received three comments, two from manufacturers and one from a government agency. All of the comments supported the affirmation of GRAS status for use of the oils in food.

FDA affirmed that partially hydrogenated menhaden oil (with an iodine number not more than 85) and fully hydrogenated menhaden oil are GRAS in the *Federal Register* of September 15, 1989 (54 FR 38219). These oils were affirmed as GRAS based on the chemical similarity between these oils and partially hydrogenated common edible vegetable oils, and on the established history of use in Europe

DEPARTMENT OF HEALTH AND HUMAN SERVICES**Food and Drug Administration****21 CFR Part 184****[Docket No. 86G-0289]****Substances Affirmed as Generally Recognized as Safe: Menhaden Oil****AGENCY:** Food and Drug Administration, HHS.**ACTION:** Final rule.

SUMMARY: The Food and Drug Administration (FDA) is affirming that menhaden oil is generally recognized as safe (GRAS) as a direct human food ingredient with specific limitations. The agency is also affirming that partially

of these oils in margarine and shortening (54 FR 38219 at 38222).

Pending further evaluation, the agency deferred its decision on menhaden oil that has not been hydrogenated, because this oil contains high levels of the *omega*-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are known to have physiologic effects, for example, effects on blood clotting (54 FR 38219). The agency's evaluation is now complete.

I. Basis for GRAS Status

Under section 201(s) of the act (21 U.S.C. 321(s)) and § 170.30 (21 CFR 170.30), general recognition of safety may be based only on the views of experts qualified by scientific training and experience to evaluate the safety of substances added to food. The basis of such views may be either: (1) Scientific procedures or, (2) in the case of a substance used in food prior to January 1, 1958, experience based on common use in food. General recognition of safety based upon scientific procedures requires the same quantity and quality of scientific evidence as is required to obtain approval of a food additive and ordinarily is to be based upon published studies, which may be corroborated by unpublished studies and other data and information (§ 170.30(b)). The petitioner relies upon scientific procedures to establish that menhaden oil is GRAS, because the oil has no history of common use as a food ingredient prior to 1958.

II. Identity

Menhaden oil is a refined marine oil that is derived from menhaden fish (*Brevoortia* species). It consists primarily of triglycerides, with small amounts of monoglycerides and diglycerides. The triglycerides are esters of glycerol and fatty acids with chains of 14 to 22 carbon atoms. Menhaden oil differs from edible vegetable oils and animal fats in its high proportion of polyunsaturated fatty acids with 4, 5 and 6 double bonds (about 25 percent). The mean percentages for these polyunsaturated fatty acids in menhaden oil are C18:4 (2.3 percent), C20:4 (2.0 percent), C20:5 (13.1 percent), C22:5 (2.5 percent) and C22:6 (6.7 percent).¹ C20:5 and C22:6 are EPA and DHA, respectively, and are the major source of *omega*-3 fatty acids from fish oil. (*Omega*-3 fatty acids refer to fatty acids with the first double bond

occurring at the third carbon from the methyl (or *omega*) end of the fatty acid.) Menhaden oil also contains about 33 percent saturated fatty acids and about 31 percent monounsaturated fatty acids.

III. Manufacturing Process

Menhaden, a plankton-feeding fish, is harvested commercially from the Gulf of Mexico and northward along the Atlantic coast of the United States. The fish is less than 12 inches long and less than a pound in weight. To produce menhaden oil, the fish is cooked whole at about 96 °C for 8 to 10 minutes to coagulate the protein and rupture the fat cells. The cooked fish is then pressed and the liquid is centrifuged to separate the oil and aqueous phases. Crude oil is then shipped to food companies for further processing, which may include storage (winterization), degumming, neutralization, bleaching, deodorization, and hydrogenation.

IV. Previous Evaluations

Data in the petition indicate that ingestion of EPA and DHA from fish oils can have a significant effect on bleeding time (the time taken for bleeding from a standardized skin wound to cease) and other physiological effects, as discussed below. Because of the potential safety concerns raised by these effects, and because there are no food oils in the food supply containing significant amounts of EPA and DHA, the agency contracted with the Mitre Corp. to perform an independent analysis of the scientific literature on the safety of menhaden oil. The Mitre Corp. issued, in April 1989, a report entitled, "Health Effects of Refined Menhaden Oil." (Copies are available from the National Technical Information Service, Order No. PB89-182398, price code A08.)

The report stated that:

[a]n increase in bleeding time is the only prominent health effect observed in humans that has been firmly established as a consequence of fish oil ingestion. This effect has been reported anecdotally in the Eskimo population and consistently observed in studies of healthy human subjects with a daily intake of 3 g [grams] of *omega*-3 fatty acids. The magnitude of the effect at this low dose is not a cause for alarm, but a lack of systematic dose-response data precludes prediction of the severity of the effect at higher daily intakes. (Pages 7-1 and 7-2 of the report.)

In addition, the Nutrition Labeling and Education Act of 1990 required FDA to evaluate health claims for 10 nutrient-disease relationships, including the relationship of *omega*-3 fatty acids and heart disease. The agency evaluated the claim that consumption of *omega*-3 fatty acids is associated with a decreased risk of coronary heart disease

under the standard set forth in section 403(r)(3) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 343(r)(3)): Whether, based on the totality of publicly available scientific evidence, there is significant scientific agreement, among experts qualified by scientific training and experience, that the claim for the diet-disease relationship is supported by the evidence. In the *Federal Register* of January 6, 1993 (58 FR 2682), FDA issued a final rule announcing its decision not to authorize a health claim relating to an association between *omega*-3 fatty acids and a decreased risk of coronary heart disease because it had concluded that there was not significant scientific agreement among experts that the totality of the scientific evidence supported the claim. Because the focus of that evaluation was a review of evidence concerning a possible beneficial effect of *omega*-3 fatty acids on the heart, a comprehensive review of the safety of *omega*-3 fatty acids from fish oils or other sources was not conducted. However, in the health claim final rule the agency did discuss, in addition to the potential health benefit, concerns over possible adverse effects of fish oils on bleeding time, glycemic control, and low-density lipoprotein (LDL) cholesterol. These issues are discussed below.

V. Safety Information

A. Bleeding Time

Increased bleeding time has been reported in many studies with humans whose diets were supplemented with fish oils. FDA stated in the health claim final rule that the importance of the increase in bleeding time reported in many studies with supplemental fish oils or with increased fish consumption is not clear (58 FR 2682 at 2699). Further, increases in bleeding time do not correlate with clinically significant bleeding, and there are debates regarding the clinical significance of the increase in bleeding time (Ref. 1). However, FDA considers excessive bleeding to be a safety concern, and has examined the scientific literature for evidence that consumption of fish oils may contribute to excessive bleeding.

There are more than 50 reports in the scientific literature on fish oils that include data on bleeding time. Several reports described the absence of changes in bleeding time, but did not provide data. A few studies involving substantial numbers of healthy human subjects indicated that there was no statistically significant increase in bleeding time after supplemental intakes of EPA and DHA from fish oils

¹ The first number refers to the total number of carbon atoms in the fatty acid; the second number refers to the total number of double bonds.

in daily amounts of 3.0 g or less (Refs. 3 through 6). Other studies with fewer human subjects, but in which the total diet was carefully controlled, also revealed that daily intakes of 3.0 g or less of EPA and DHA in fish oils did not increase bleeding time (Refs. 7 and 8).

However, two studies described increases in bleeding time that were reported to be statistically significant. Subjects in the studies consumed about 3.0 g per person per day (p/d) EPA and DHA from fish oils. Mortensen et al. (Ref. 9), in a crossover, double-blind, placebo-controlled study among 20 normal, healthy males, showed that consumption of slightly more than 3.0 g/d of EPA and DHA in fish oil capsules for 4 weeks produced a small but statistically significant increase (16 percent) in median bleeding time; however, both the mean and 75th percentile bleeding times were well within the normal range. Harris and Windsor (Ref. 10) reported that consumption of fish oil containing 2.2 g/d of EPA and DHA also produced a small (15 percent) but statistically significant increase in bleeding time, but this increase was also within the normal range.

Studies in which greater daily amounts (higher than 3.0 g/p/d) of fish oils were fed often reported statistically significant increases in bleeding time (Refs. 11 through 22). In some of those studies, use of fish oils resulted in substantial prolongation of bleeding time outside the normal range, as indicated by the standard deviations reported (Refs. 8, 12, 18, 21, and 22). However, the pre-treatment bleeding times in those studies were also beyond the normal range, making it difficult to evaluate the effect of fish oils on bleeding time. In other studies, the increase in bleeding time after daily intakes of more than 3.0 g of EPA and DHA is difficult to interpret meaningfully because of the small number of subjects tested (Refs. 23 through 27).

Studies have also been carried out with subjects who had evidence of coronary heart disease or risk factors for coronary heart disease. After intake of 3.2–6.0 g/p/d of EPA and DHA in fish oils, many of these subjects showed increased bleeding time (Refs. 20, and 28 through 33). However, none of the studies reported evidence that the prolonged bleeding time was clinically significant. In those cases where the effect of fish oils in angioplasty or bypass surgery patients (a total of 520 patients fed supplemental fish oil) was studied, excessive bleeding was not reported even though acetylsalicylic acid (aspirin), which itself greatly

prolongs bleeding time, was used concurrently (Refs. 34 through 40). One large study that used a dose of 6 g/p/d EPA and DHA in fish oils did report four cases of increased bleeding in the fish oil group (of 124 treated) versus none in the placebo group, but the difference in rates of occurrences between the two groups was not statistically significant (Ref. 40).

In summary, the totality of the scientific evidence demonstrates that when consumption of fish oils is limited to 3 g/p/d or less of EPA and DHA, there is no significant risk for increased bleeding time beyond the normal range. A report from an industry-sponsored roundtable discussion on the topic of fish oils and bleeding time (Ref. 2) also supports the conclusion that EPA and DHA are safe at intake levels at or below 3 g/p/d. On the other hand, amounts of fish oils providing more than 3 g/d of EPA and DHA have generally been found to produce increases in bleeding time that are statistically significant. At this time, there are insufficient data to evaluate the clinical significance of this finding. Because of the lack of data and because of the potential risk of excessive bleeding in some individuals with intakes at higher levels, FDA concludes that the safety of menhaden oil is generally recognized only at levels that limit intake of EPA and DHA to 3 g/p/d.

B. Glycemic Control

Some studies on non-insulin-dependent diabetics have reported increased glucose levels when large amounts of fish oils (4.5 to 8.0 g/p/d) were used in the diet. In the health claim final rule, FDA discussed the possible adverse effects of fish oil consumption on glycemic control among diabetics and stated that such effects were a safety concern (58 FR 2682 at 2704 through 2705). FDA concluded in that document that the effects of fish oils on blood glucose appear to depend on the amount of fish oils fed, based on review of a number of studies (58 FR 2682 at 2705). One study found no change in fasting blood glucose levels among type-II [non-insulin-dependent] diabetics treated with 3.0 g/d EPA plus DHA for 2 weeks (Ref. 41). Two other studies that used 3 g/d EPA plus DHA for 6 weeks (Ref. 42) and 2.7 g/d EPA plus DHA for 8 weeks (Ref. 43) found only transient increases in blood glucose halfway through their respective supplementation periods. Another study (Ref. 44) that used 3.0 g/d EPA plus DHA for 3 weeks found comparable increases in fasting blood glucose when either fish oil or safflower oil was fed, so the increase cannot be

attributed specifically to *omega*-3 fatty acids. A study that compared the effects of fish oil and olive oil (Ref. 45) fed 3 g/d of EPA plus DHA and did not find a difference in fasting glucose or glycosylated hemoglobin after fish oil supplementation compared to baseline; they did find a significant difference compared to the olive oil treatment, which produced changes in the opposite direction from fish oil. Studies on type II diabetics that reported increased glucose used higher amounts (4.5 to 8 g/d) of *omega*-3 fatty acids (Refs. 46 through 49).

Based on the available information, FDA concludes that consumption of EPA and DHA in fish oils at 3 g/p/d by diabetics has no clinically significant effect on glycemic control, although higher amounts of EPA and DHA (4.5 g/p/d and above) remain of concern. Therefore, FDA concludes that 3 g/p/d of EPA and DHA is a safe level with respect to glycemic control.

C. LDL Cholesterol

In the health claim final rule, FDA noted that many studies on hypertriglyceridemic or hypercholesterolemic subjects, and some studies on normal subjects, reported an increase in LDL cholesterol or apo B (apolipoprotein B, a principal component of LDL) following fish oil supplementation (58 FR 2682 at 2705). Because increases in LDL cholesterol predict increased risk of coronary heart disease, FDA recently reevaluated those studies, as well as newer studies published since the health claim final rule, to address the question of whether 3 g/p/d of EPA and DHA derived from menhaden oil is generally recognized as a safe level with respect to its effect on LDL cholesterol. The agency considered the reported effects of fish oil on LDL cholesterol levels in healthy persons with normal cholesterol levels, as well as in persons with diabetes mellitus, hypertension, abnormal blood lipid levels, and cardiovascular disease.

As a result of its reevaluation, FDA found that although reported study results are variable, there appears to be a trend toward increased LDL cholesterol values with increased fish oil consumption in all population subgroups, with the magnitude of the increase appearing greater and more consistent in populations with abnormal blood lipid levels, hypertension, diabetes, and cardiovascular disease.

In the health claims final rule, FDA noted that because most reports of increased LDL were in studies where large amounts of fish oil were given (i.e., 5 g or more per day of EPA plus DHA), any safety concern relating to

changes in LDL cholesterol might be suitably addressed by restricting the intake of DHA and EPA (58 FR 2682 at 2705). As discussed below, the petitioner has suggested maximum use levels of menhaden oil for each food category in which menhaden oil can be used. Based on these levels, FDA has determined that the mean intake of menhaden oil, if menhaden oil were to be used at the maximum allowable level in all permitted food categories, would be less than 3 g of DHA and EPA per day. Further, menhaden oil would substitute for other dietary fats, some of which have similar effects on LDL cholesterol. Based on its evaluation, the agency concludes that the petitioned levels of menhaden oil are safe with respect to the effect on LDL cholesterol.

VI. Consumer Exposure

In September 1993, the petitioner amended the petition to include maximum use levels for menhaden oil in various food categories. Based on these levels, FDA estimated that the mean exposure to EPA and DHA from the use of menhaden oil in all food categories would be 2.8 g/p/d (Ref. 50). Although the petition originally included all potential food uses of menhaden oil, the petitioner subsequently requested that the use of menhaden oil in infant formula be withdrawn from consideration. Therefore, the exposure estimate does not include this potential use of menhaden oil.

VII. Iodine Numbers of Oils from Menhaden

When FDA affirmed hydrogenated and partially hydrogenated menhaden oils as GRAS based on their pre-1958 history of safe use in food, the agency included in the regulation a specification that the iodine number for partially hydrogenated menhaden oil be no more than 85. (Iodine number is a measure of the unsaturation of fats and oils, expressed in terms of centigrams of iodine absorbed per gram of sample.) The iodine number limit of 85 was chosen then because menhaden oil with an iodine number greater than 85 is not considered hardened, and only hardened oil had a documented history of common use in food before 1958 (54 FR 38219 at 38222). Moreover, corroborative toxicological studies submitted in the petition used oil with an iodine number no more than 85 (54 FR 38219 at 38222). The iodine number limit of 85 also ensured that the partially hydrogenated menhaden oil affirmed as GRAS at that time would contain no more than traces of EPA and DHA, and thus would not significantly

increase the dietary intake of these substances, pending completion of the agency's evaluation of the safety of DHA and EPA as part of its review of the GRAS status of menhaden oil. By specifying this upper limit, the agency deferred its decision on the GRAS status of partially hydrogenated menhaden oil with an iodine number above 85.

The agency now concludes (as stated below), based on scientific procedures, that menhaden oil is GRAS, provided that daily intakes of EPA and DHA from menhaden oil do not exceed 3 g/p/d. The petitioner has provided information demonstrating that partially hydrogenated menhaden oil may have an iodine number up to 119. The agency finds that the use of partially hydrogenated menhaden oil with an iodine number up to 119 under conditions specified in current 21 CFR 184.1472 will not cause the total exposure to EPA and DHA from all types of menhaden oil to exceed 3 g/p/d (Ref. 50). Therefore, FDA concludes that partially hydrogenated menhaden oil with an iodine number between 86 and 119 is GRAS based on scientific procedures, and is raising the iodine number limit in the regulation for partially hydrogenated menhaden oil to 119. With this change, the iodine number range for partially hydrogenated menhaden oil will be 11 through 119 instead of 11 through 85.

The effect of the change in the iodine number range for partially hydrogenated menhaden oil will be to affirm as GRAS a substance that was not previously affirmed as GRAS (i.e., partially hydrogenated menhaden oil with an iodine number between 86 and 119), rather than to amend the specifications for a substance already affirmed as GRAS. Even if the change in the iodine number range is characterized as an amendment, however, the Administrative Procedure Act (5 U.S.C. 553(b)(3)(B)) permits an agency to amend a regulation without notice and comment procedures when the agency for good cause finds that such procedures are impracticable, unnecessary, or contrary to the public interest. Because notice of the filing of a petition seeking GRAS affirmation of menhaden oil and partially hydrogenated menhaden oil was given (51 FR 27461), and an opportunity for public comment on all issues relating to the petition, including iodine number ranges, was provided at that time, FDA finds that separate, additional notice and comment procedures on the specific issue of the iodine number range for partially hydrogenated menhaden oil are unnecessary. Therefore, the agency finds that there is good cause to proceed

to final action without an opportunity for additional public comment on this issue.

VIII. Conclusions

FDA has evaluated the information in the petition and many published articles in scientific journals, along with other relevant information. Based on this evaluation, the agency finds that the use of menhaden oil as a direct food ingredient is safe, provided that daily intakes of EPA and DHA from menhaden oil do not exceed 3 g/p/d. As noted in section VI of this document, the petitioned uses of menhaden oil incorporate maximum use levels for menhaden oil in specific food categories to ensure that daily intakes of EPA and DHA from menhaden oil do not exceed 3 g/p/d. FDA has further determined that the many pertinent published human clinical studies provide an adequate basis to conclude that the safety of the petitioned uses of menhaden oil is generally recognized among the community of experts qualified by scientific training and experience to evaluate the safety of food ingredients. Therefore, the agency is affirming that the use of menhaden oil as a direct human food ingredient is GRAS with specific limitations (21 CFR 184.1(b)(2)). This GRAS affirmation is based on scientific procedures (21 CFR 170.30(b)). To ensure that only food-grade menhaden oil is used in food, FDA is including appropriate specifications in the regulation.

FDA further concludes, based on scientific procedures, that partially hydrogenated menhaden oil with an iodine number between 86 and 119 is GRAS with no limitation other than current good manufacturing practice. Therefore, the agency is increasing the iodine number limit for partially hydrogenated menhaden oil to 119.

IX. Environmental Impact

The agency is affirming that menhaden oil is generally recognized as safe (GRAS) as a direct human food ingredient with specific limitations. The agency is also affirming that partially hydrogenated menhaden oil with an iodine number between 86 and 119 is GRAS as a direct human food ingredient with no limitation other than current good manufacturing practice.

The agency has carefully considered the potential environmental effects of these actions. FDA has concluded that these actions will not have a significant impact on the human environment, and that an environmental impact statement is not required. The agency's finding of no significant impact and the evidence supporting that finding, contained in an

environmental assessment, may be seen in the Dockets Management Branch (HFA-305), Food and Drug Administration, 12420 Parklawn Dr., rm. 1-23, Rockville, MD 20857, between 9 a.m. and 4 p.m., Monday through Friday.

X. Analysis of Impacts

FDA has examined the economic implications of the final rule as required by Executive Order 12866 and the Regulatory Flexibility Act (5 U.S.C. 601-612). Executive Order 12866 directs agencies to assess all costs and benefits of available regulatory alternatives and, when regulation is necessary, to select the regulatory approach that maximizes net benefits (including potential economic, environmental, public health and safety effects; distributive impacts; and equity). Executive Order 12866 classifies a rule as significant if it meets any one of a number of specified conditions, including having an annual effect on the economy of \$100 million or adversely affecting in a material way a sector of the economy, competition, or jobs, or if it raises novel legal or policy issues. If a rule has a significant economic impact on a substantial number of small entities, the Regulatory Flexibility Act requires agencies to analyze regulatory options that would minimize the economic impact of that rule on small entities.

FDA finds that this final rule is not a significant rule as defined by Executive Order 12866. This final rule recognizes the applicability of a statutory exemption. The impact of the rule is to remove uncertainty about the regulatory status of the petitioned substance. Accordingly, under the Regulatory Flexibility Act, 5 U.S.C. 605(b), the Commissioner of Food and Drugs certifies that this final rule will not have a significant economic impact on a substantial number of small entities (Ref. 51).

XI. Effective Date

As this rule recognizes an exemption from the food additive definition in the Federal Food, Drug, and Cosmetic Act, and from the approval requirements applicable to food additives, no delay in effective date is required by the Administrative Procedure Act (5 U.S.C. 553(d)). The rule will therefore be effective immediately (5 U.S.C. 553(d)(1)).

XII. References

The following information has been placed on display with the Dockets Management Branch (address above), and may be seen by interested persons

between 9 a.m. and 4 p.m., Monday through Friday.

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50. Memorandum, October 19, 1993, Michael DiNovi, FDA, Washington, DC to Lawrence Lin, FDA, Washington, DC.

51. Memorandum, May 16, 1997, William Hubbard, Associate Commissioner for Policy Coordination, FDA, Rockville, MD to Lawrence Lin, FDA, Washington, DC.

List of Subjects in 21 CFR Part 184

Food additives, Food ingredients, Incorporation by reference.

Therefore, under the Federal Food, Drug, and Cosmetic Act and under authority delegated to the Commissioner of Food and Drugs, and redelegated to the Director, Center for Food Safety and Applied Nutrition, 21 CFR part 184 is amended as follows:

PART 184—DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE

1. The authority citation for 21 CFR part 184 continues to read as follows:

Authority: Secs. 201, 402, 409, 701 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 321, 342, 348, 371).

2. Section 184.1472 is revised to read as follows:

§ 184.1472 Menhaden oil.

(a) *Menhaden oil*. (1) Menhaden oil is prepared from fish of the genus *Brevoortia*, commonly known as menhaden, by cooking and pressing. The resulting crude oil is then refined using the following steps: Storage (winterization), degumming (optional), neutralization, bleaching, and deodorization. Winterization may separate the oil and produce a solid fraction.

(2) Menhaden oil meets the following specifications:

(i) *Color and state*, Yellow liquid to white solid.

(ii) *Odor*, Odorless to slightly fishy.

(iii) *Saponification value*, Between 180 and 200 as determined by the American Oil Chemists' Society Official Method Cd 3-25—"Saponification Value" (reapproved 1989), which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies of this publication are available from the Office of Premarket Approval, Center for Food Safety and Applied Nutrition (HFS-200), Food and Drug Administration, 200 C St. SW., Washington, DC 20204, or available for inspection at the Center for Food Safety and Applied Nutrition's Library, Food and Drug Administration, 200 C St. SW., rm. 3321, Washington DC, or at the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

(iv) *Iodine number*, Not less than 120 as determined by the American Oil Chemists' Society Recommended Practice Cd 1d-92—"Iodine Value of Fats and Oils, Cyclohexane—Acetic Acid Method," which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. The availability of this incorporation by reference is given in paragraph (a) (2) (iii) of this section.

(v) *Unsaponifiable matter*, Not more than 1.5 percent as determined by the American Oil Chemists' Society Official Method Ca 6b-53—"Unsaponifiable Matter" (reapproved 1989), which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. The availability of this incorporation by reference is given in paragraph (a) (2) (iii) of this section.

(vi) *Free fatty acids*, Not more than 0.1 percent as determined by the American Oil Chemists' Society Official Method Ca 5a-40—"Free Fatty Acids"

(reapproved 1989), which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. The availability of this incorporation by reference is given in paragraph (a) (2) (iii) of this section.

(vii) *Peroxide value*. Not more than 5 milliequivalents per kilogram of oil as determined by the American Oil Chemists' Society Official Method Cd 8-53—"Peroxide Value, Acetic Acid—Chloroform Method" (updated 1992) or Recommended Practice Cd 8b-90—"Peroxide Value, Acetic Acid—Isooctane Method" (updated 1992), which are incorporated by reference in accordance with 5 U.S.C. 552(a) and 1

CFR part 51. The availability of this incorporation by reference is given in paragraph (a)(2)(iii) of this section.

(viii) *Lead*. Not more than 0.1 part per million as determined by the American Oil Chemists' Society Official Method Ca 18c-91—"Determination of Lead by Direct Graphite Furnace Atomic Absorption Spectrometry" (revised 1992), which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. The availability of this incorporation by reference is given in paragraph (a)(2)(iii) of this section.

(ix) *Mercury*. Not more than 0.5 part per million as determined by the method entitled "Biomedical Test

Materials Program: Analytical Methods for the Quality Assurance of Fish Oil," published in the "NOAA Technical Memorandum NMFS-SEFC-211," F. M. Van Dolah and S. B. Galloway, editors, National Marine Fisheries Service, U. S. Department of Commerce, pages 71-88, November, 1988, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. The availability of this incorporation by reference is given in paragraph (a)(2)(iii) of this section.

(3) In accordance with § 184.1(b)(2), the ingredient may be used in food only within the following specific limitations:

Category of food	Maximum level of use in food (as served)
Cookies, crackers, § 170.3(n)(1) of this chapter.	5.0 percent
Breads, rolls (white & dark), § 170.3(n)(1) of this chapter.	1.0 percent
Fruit pies, custard pies, § 170.3(n)(1) of this chapter.	7.0 percent
Cakes, § 170.3(n)(1) of this chapter.	10.0 percent
Cereals, § 170.3(n)(4) of this chapter.	4.0 percent
Fats, oils, § 170.3(n)(12) of this chapter, but not in infant formula.	20.0 percent
Yogurt, § 170.3(n)(31) of this chapter.	4.0 percent
Cheese products, § 170.3(n)(5) of this chapter.	5.0 percent
Frozen dairy products, § 170.3(n)(20) of this chapter.	5.0 percent
Meat products, § 170.3(n)(29) of this chapter.	10.0 percent
Egg products, § 170.3(n)(11) of this chapter.	5.0 percent
Fish products, § 170.3(n)(13) of this chapter.	20.0 percent
Condiments, § 170.3(n)(8) of this chapter.	5.0 percent
Soup mixes, § 170.3(n)(40) of this chapter.	3.0 percent
Snack foods, § 170.3(n)(37) of this chapter.	5.0 percent
Nut products, § 170.3(n)(32) of this chapter.	5.0 percent
Gravies, sauces, § 170.3(n)(24) of this chapter.	5.0 percent

(b) *Hydrogenated and partially hydrogenated menhaden oils*. (1) Partially hydrogenated and hydrogenated menhaden oils are prepared by feeding hydrogen gas under pressure to a converter containing crude menhaden oil and a nickel catalyst. The reaction is begun at 150 to 160 °C and after 1 hour the temperature is raised to 180 °C until the desired degree of hydrogenation is reached. Hydrogenated menhaden oil is fully hydrogenated.

(2) Partially hydrogenated and hydrogenated menhaden oils meet the following specifications:

- (i) *Color*. Opaque white solid.
- (ii) *Odor*. Odorless.
- (iii) *Saponification value*. Between 180 and 200.

(iv) *Iodine number*. Not more than 119 for partially hydrogenated menhaden oil and not more than 10 for fully hydrogenated menhaden oil.

(v) *Unsaponifiable matter*. Not more than 1.5 percent.

(vi) *Free fatty acids*. Not more than 0.1 percent.

(vii) *Peroxide value*. Not more than 5 milliequivalents per kilogram of oil.

(viii) *Nickel*. Not more than 0.5 part per million.

(ix) *Mercury*. Not more than 0.5 part per million.

(x) *Arsenic (as As)*. Not more than 0.1 part per million.

(xi) *Lead*. Not more than 0.1 part per million.

(3) Partially hydrogenated and hydrogenated menhaden oils are used as edible fats or oils, as defined in § 170.3(n)(12) of this chapter, in food at levels not to exceed current good manufacturing practice.

(4) If the fat or oil is fully hydrogenated, the name to be used on the label of a product containing it shall include the term "hydrogenated," or if it is partially hydrogenated, the name shall include the term "partially hydrogenated," in accordance with § 101.4(b)(14) of this chapter.

Dated: May 22, 1997.

Fred R. Shank,

Director, Center for Food Safety and Applied Nutrition.

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Distribution, interconversion, and dose response of n-3 fatty acids in humans¹⁻⁴

Linda M Arterburn, Eileen Bailey Hall, and Harry Oken

ABSTRACT

n-3 Fatty acids have important visual, mental, and cardiovascular health benefits throughout the life cycle. Biodistribution, interconversion, and dose response data are reviewed herein to provide a basis for more rational n-3 dose selections. Docosahexaenoic acid (DHA) is the principal n-3 fatty acid in tissues and is particularly abundant in neural and retinal tissue. Limited storage of the n-3 fatty acids in adipose tissue suggests that a continued dietary supply is needed. A large proportion of dietary α -linolenic acid (ALA) is oxidized, and because of limited interconversion of n-3 fatty acids in humans, ALA supplementation does not result in appreciable accumulation of long-chain n-3 fatty acids in plasma. Eicosapentaenoic acid (EPA) but not DHA concentrations in plasma increase in response to dietary EPA. Dietary DHA results in a dose-dependent, saturable increase in plasma DHA concentrations and modest increases in EPA concentrations. Plasma DHA concentrations equilibrate in approximately 1 mo and then remain at steady state throughout supplementation. DHA doses of ≈ 2 g/d result in a near maximal plasma response. Both dietary DHA and EPA reduce plasma arachidonic acid concentrations. Tissue contents of DHA and EPA also increase in response to supplementation with these fatty acids. Human milk contents of DHA are dependent on diet, and infant DHA concentrations are determined by their dietary intake of this fatty acid. We conclude that the most predictable way to increase a specific long-chain n-3 fatty acid in plasma, tissues, or human milk is to supplement with the fatty acid of interest. *Am J Clin Nutr* 2006;83(suppl):1467S-76S.

KEY WORDS Docosahexaenoic acid, eicosapentaenoic acid, α -linolenic acid, n-3 fatty acids, human dose response

INTRODUCTION

The n-3 fatty acids make up a family of essential fats that humans are unable to synthesize de novo. The parent 18-carbon fatty acid, α -linolenic acid (ALA; 18:3n-3), is present in various vegetable oils, such as flaxseed, linseed, canola, and soy oils. Americans on average consume ≈ 1.3 g ALA/d (1-3). ALA can be metabolically converted to the various long-chain n-3 fatty acids, including eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). However, enzymatic conversion efficiencies vary considerably among species and appear to be relatively inefficient in humans. The principal long-chain n-3 fatty acids in the diet are DHA and EPA. Algae are the primary producers of DHA and EPA in the ecosystem, and several refined algal oils are rich sources of DHA. Fish consume algae and therefore are rich in DHA and EPA. With limited intake

of marine foods in the United States, combined intake of DHA and EPA is estimated at only ≈ 100 mg/d (1, 2).

Fatty acids of the n-3 family, particularly the long-chain n-3 fatty acids, are important nutrients throughout the life cycle. Infants require DHA for visual and cognitive development (4-6), and in children, cardiovascular benefits have been attributed to long-chain n-3 fatty acids (7, 8). DHA and EPA are important for the prevention of cardiovascular disease and resulted in decreased cardiac mortality in a large secondary prevention study (9). Recent epidemiologic and preclinical studies also suggest that DHA may protect against Alzheimer disease and other types of dementia (10-12), and long-chain n-3 fatty acids may protect against advanced age-related macular degeneration (13), which suggests a continued role of these fats in brain and eye health in adults and the elderly.

The potential health benefits of n-3 fatty acids have been examined in many clinical trials. These studies, however, generally used combinations of n-3 fatty acids, especially DHA plus EPA, which makes it difficult to discern specific roles and health benefits of the individual n-3 fatty acids. A main focus of this symposium is to provide more clarity on the functional role of the individual n-3 fatty acids. The recent availability of pure sources of DHA and EPA has helped to accelerate this research. In addition, many clinical studies used high pharmacologic doses of n-3 fatty acids to ensure maximal therapeutic effects, but failed to provide dose ranging information, particularly with respect to lower intakes compatible with nutrients in foods. The purpose of the present review is to explore the tissue distribution, interconversion, and dose effects of the specific n-3 fatty acids on fatty acid levels in human blood and tissues and thereby provide guidance for developing n-3 dose selection and recommendations.

TISSUE DISTRIBUTION OF n-3 FATTY ACIDS

n-3 Fatty acids are present in cell membranes and are incorporated primarily into phospholipids, as well as sphingolipids and plasmalogens. These fatty acids, particularly the more highly

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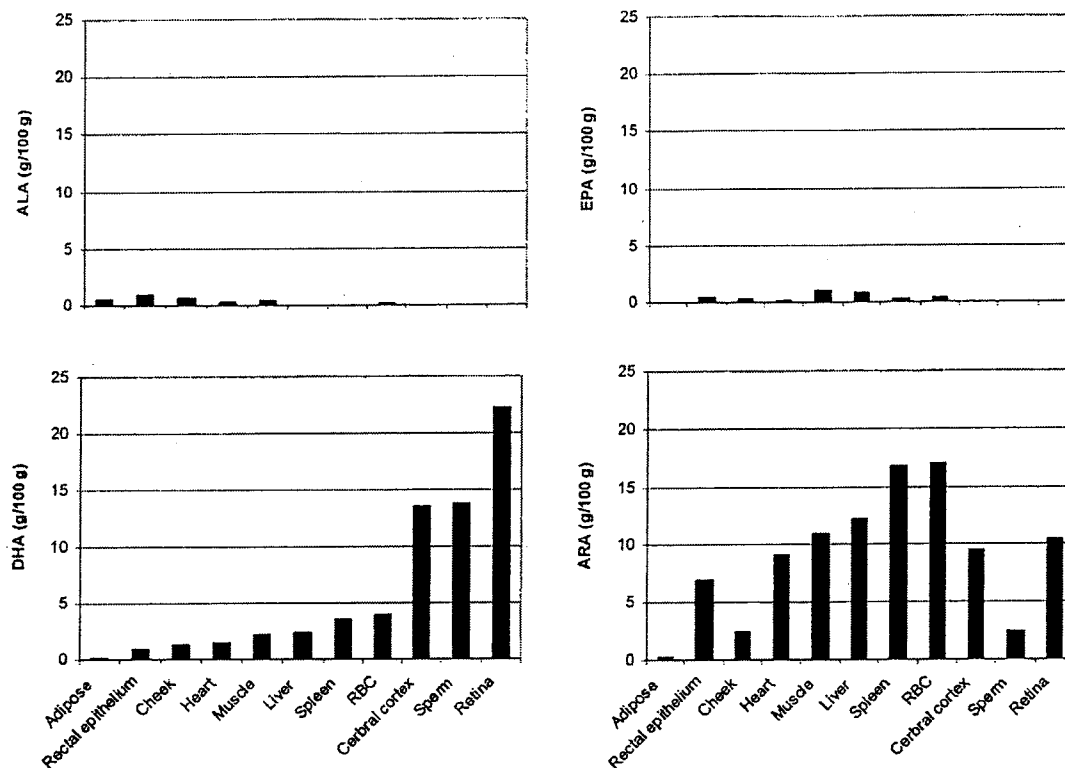


FIGURE 1. Cross-study analysis of fatty acid concentrations (g/100 g of total fatty acids) in tissues from adults from the United States, Canada, Australia, or Europe. References: adipose tissue (21), rectal epithelium (22), muscle (23), liver and spleen (24), heart and cheek (25), red blood cell (26), cerebral cortex (27), sperm (28), and peripheral retina (29). ALA, α -linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; RBC, red blood cell.

unsaturated long-chain $n-3$ fatty acids, can influence the biophysical properties of membranes (eg, fluidity, thickness, and deformability) and therefore affect activity of transmembrane proteins (14). DHA, as the most unsaturated fatty acid in membranes, is highly flexible within the membrane and is particularly effective at accommodating transitional changes associated with transmembrane protein activation (15, 16). DHA, EPA, and ALA, as well as the $n-6$ fatty acid arachidonic acid (ARA), compete for the $sn-2$ position on membrane phospholipids. The relative proportion of these fatty acids also determines their availability after phospholipase cleavage as substrates for cyclooxygenases and lipoxygenases, and hence the balance of eicosanoids and other antiinflammatory autoids, such as resolvins (17). These fatty acids are also ligands for nuclear receptors such as peroxisome proliferator-activated receptors and retinoid X receptor, and can therefore influence gene regulation (18–20). Thus, the overall membrane fatty acid composition can have a large impact on cell and organ function as well as a wide variety of biological processes.

The proportions of the $n-3$ fatty acids ALA, EPA and DHA, as well as of the $n-6$ fatty acid ARA for comparison, found in various organs of adults from the United States, Canada, Europe, or Australia are depicted in **Figure 1**. DHA is the most abundant $n-3$ fatty acid in membranes and is present in all organs. It is also the most variable among organs and is particularly abundant in neural tissue, such as brain and retina. Only minute quantities of ALA and EPA are generally present in tissues, and DHA generally exceeds EPA 5- to 30-fold in most organs. However, DHA

is several hundred-fold more abundant than EPA in brain and retina. For comparison, ARA is also relatively abundant in most tissues but has a distribution distinct from that of DHA.

In adipose tissue, where fatty acids are stored as triacylglycerol, linoleic acid (LA; 18:2 $n-6$) is the most abundant polyunsaturated fatty acid at ≈ 12 –16% of fatty acids, and ALA is the predominant $n-3$ fatty acid at $\approx 1\%$ (21, 30, 31). Only very small amounts of DHA or EPA are present in adipose tissue (21, 30, 31), which suggests a limited storage capacity of these long-chain $n-3$ fatty acids and implies the need for a continuous supply through the diet.

In healthy North Americans, DHA generally constitutes $\approx 4\%$ of total lipid in red blood cells (26, 32, 33). The mean red blood cell DHA content in healthy persons who do not regularly take DHA supplements as measured in our laboratory ($n = 284$) is 4.0% of total fatty acids, with a normal range of 1.9% to 7.9%. Mean plasma or serum phospholipid DHA contents have been reported in the range of 2.5% to 3.4% of fatty acids in healthy adults in North America (28, 32, 34). The mean plasma phospholipid DHA content in healthy individuals measured in our laboratory is 3.5% of total fatty acids ($n = 294$), with a normal range of 1.5% to 7.5%.

METABOLIC INTERCONVERSION OF $n-3$ FATTY ACIDS

The conversion of ALA to EPA and DHA occurs primarily in the liver in the endoplasmic reticulum and involves a series of elongation enzymes that sequentially add 2-carbon units to the



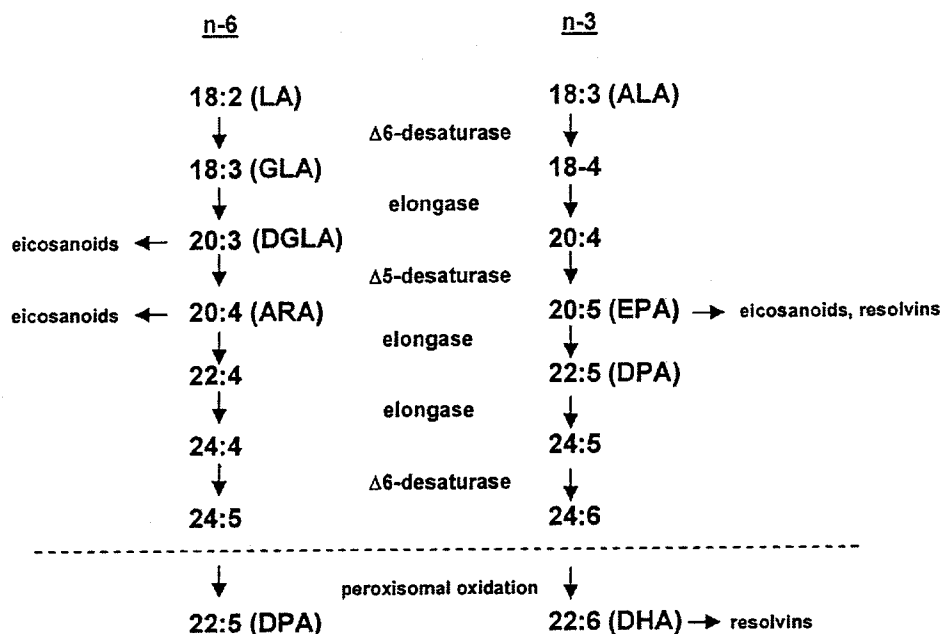


FIGURE 2. Biochemical pathway for the interconversion of n-6 and n-3 fatty acids. ALA, α -linolenic acid; ARA, arachidonic acid; DGLA, dihomo- γ -linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, γ -linolenic acid; LA, linoleic acid.

fatty acid backbone and desaturation enzymes that insert double bonds into the molecules (Figure 2). The final conversion of ALA to DHA requires a translocation to the peroxisome for a β -oxidation reaction. Because the same enzymes involved in n-3 synthesis are responsible for conversion of the n-6 fatty acid LA to ARA, background diet can influence the conversion of these fatty acids. The intake of LA in the American diet, which is ≈ 15 g/d, is approximately an order of magnitude greater than the intake of ALA (1-3).

Several investigators have performed detailed studies of the *in vivo* conversion of ALA to its long-chain n-3 derivatives EPA, docosapentaenoic acid (DPA), and DHA in humans by using uniformly labeled [^{13}C]- or [^2H]ALA as a tracer (reviewed in references 35 and 36). These studies have consistently shown that ≥ 15 -35% of dietary ALA is rapidly catabolized to carbon dioxide for energy (37-40), and that only a small proportion, estimated by using compartment models to be $< 1\%$, is converted to DHA (41, 42). In fact, ALA has the highest rate of oxidation among all unsaturated fatty acids (43). The fractional conversion of ALA to EPA, estimated by measuring peak or area under the curve plasma contents of the labeled fatty acids, varies between 0.3% and 8% in men, and the conversion of ALA to DHA is $< 4\%$ and often undetectable in males (39, 40, 44, 45). Conversion of ALA to long-chain n-3 fatty acids appears to be more efficient in women: up to 21% is converted to EPA and up to 9% is converted to DHA (38), with a concomitant reduction in the rate of ALA oxidation ($\approx 22\%$ compared with $\approx 33\%$ in men) (38-40). The conversion of DPA to DHA is the rate-limiting step in the conversion of ALA to DHA, and dietary DHA and EPA down-regulate this step by 70% (41). Others have also shown that dietary EPA and DHA reduce the conversion of ALA to long-chain n-3 fatty acids (37, 39). Diets high in LA may influence, through substrate competition and inhibition of Δ^6 desaturase enzyme, the metabolism of the n-3 fatty acids. Emken et al (44) showed that a diet high in LA reduces the conversion of ALA to

its long-chain derivatives by 40%, with a net reduction in long-chain n-3 fatty acid accumulation of 70%. Diets high in ALA appear to increase the rate of ALA oxidation, limiting its accumulation in plasma and reducing its conversion rate to EPA and DHA (37). Considerable variability in the conversion rates among individuals has been reported, even when the subjects have similar background diets (44). This interindividual variability, along with modest ALA intakes and high amounts of LA in the American diet, suggests that ALA cannot reliably replace EPA and particularly DHA in the diet.

DHA itself also serves as a substrate for metabolic retroconversion to EPA and DPA through a β -oxidation reaction. In studies in which ^{13}C -labeled DHA was fed, Brossard et al (46) calculated the retroconversion rate of DHA to EPA in humans receiving normal dietary amounts of DHA to be $\approx 1.4\%$. Human clinical data have suggested rates as high as 12% with high chronic DHA consumption (47, 48). Retroconversion of DHA to EPA is hormonally regulated and decreases in women receiving hormone replacement therapy (49).

EFFECTS OF ALA SUPPLEMENTATION ON PLASMA FATTY ACIDS

Several case studies involving n-3-deficient patients reported that intervention with ALA results in marked increases in plasma concentrations of both EPA and DHA (50, 51). In addition, vegans who consume ALA but not EPA and DHA in their diets have low but stable concentrations of DHA in plasma (52, 53). Together, these findings suggest that humans can convert meaningful quantities of ALA to EPA and DHA, particularly in the presence of a deficiency or a background of low n-6 fatty acids. However, consistent with the isotope-tracer studies, the vast majority of the ALA supplementation studies show a limited conversion of ALA to its long-chain n-3 derivatives. These studies have been performed in healthy individuals consuming typical

Western diets generally high in n-6 fatty acids. Chan et al (54), for example, studied the dose effect of ALA supplementation on plasma phospholipid n-3 fatty acid concentrations and found a small but significant dose-dependent increase in ALA and EPA concentrations in phospholipids, but no increase in DHA. Notably, a high ratio of LA to ALA in the diet reduced the conversion of ALA to EPA. Others have found increased amounts of ALA and EPA but not DHA after supplementation with 2-3 g ALA/d (55, 56).

To explore the ALA dose-response relation across a wider range of doses in a systematic fashion, we conducted a cross-study meta-regression analysis of plasma phospholipid n-3 fatty acid concentrations after ALA supplementation. Changes in plasma phospholipid fatty acid concentrations from various studies involving supplementation of healthy adults with ALA-rich oils are plotted in Figure 3. ALA supplementation with up to 14 g/d resulted in dose-dependent but modest increases in plasma ALA concentrations. Some of the observed variability, especially at low ALA doses, is attributed to differences in the amount of LA concurrently administered in the diet, because LA reduces ALA accumulation (44). Nonetheless, the dose response appears linear ($r^2 = 0.79$, $P = 0.008$). There are small increases in EPA after ALA supplementation ($r^2 = 0.49$, $P = 0.052$); however, plasma phospholipid DHA concentrations do not detectably increase after ALA supplementation with doses up to 14 g/d. This analysis suggests that, given the typical Western diet, even large doses of ALA result in only marginal changes in ALA concentrations in plasma, small increases in plasma EPA, and no effect on DHA, which is consistent with a high oxidation rate and the low fractional conversion rates discussed above. These conversion rates may be altered in the presence of a diet containing reduced amounts of n-6 fatty acids.

EFFECT OF DHA SUPPLEMENTATION ON PLASMA FATTY ACIDS

Many studies have shown that supplementation with DHA triacylglycerol or DHA ethyl esters results in increased plasma DHA concentrations (47, 49, 63-70). We have further analyzed the dose-response effect of supplemental DHA on plasma phospholipid fatty acid concentrations by using a cross-study meta-regression analysis. Plasma phospholipid fatty acid concentrations from 12 different studies (16 different supplementation groups) with doses ranging from 0.2 to 6 g DHA/d for 1 to 6 mo are shown in Figure 4. This analysis shows that plasma phospholipid DHA concentrations increase in a dose-dependent, saturable manner in response to dietary DHA, which was suggested previously by Vidgren et al (71). Plasma phospholipid DHA concentrations are highly sensitive to dietary intake of this fatty acid at doses up to ≈ 2 g/d. At doses above this amount, plasma DHA concentrations approach saturation and increase only incrementally. DHA supplementation also results in an apparent linear increase in EPA concentrations, presumably through retroconversion, with EPA concentrations increasing by ≈ 0.4 g/100 g fatty acid for each 1 g of DHA intake. There is also a concurrent dose-dependent, saturable reduction in plasma phospholipid ARA concentrations, although the ARA response is more variable among studies.

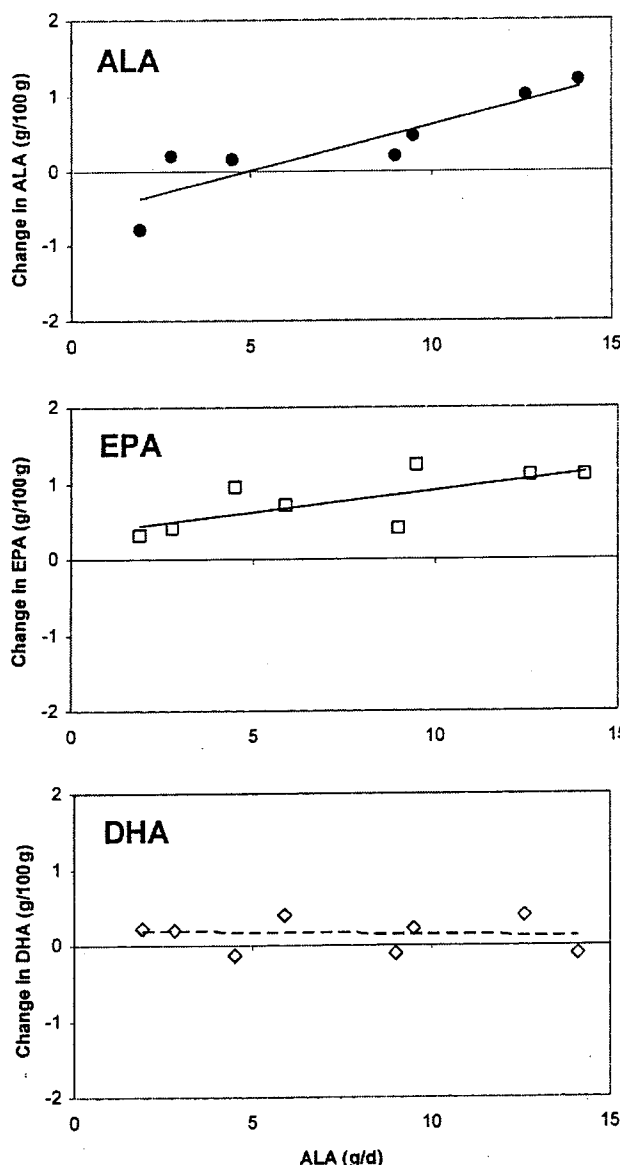


FIGURE 3. Cross-study meta-regression analysis of the effect of α -linolenic acid (ALA) supplementation of adults on plasma phospholipid ALA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) concentrations. Clinical studies involving supplementation with ALA-rich oils, which were identified through PUBMED (National Library of Medicine, Bethesda, MD), that reported both ALA intakes and plasma phospholipid fatty acid concentrations in g/100 g of fatty acids in supplementation groups of ≥ 10 healthy individuals for ≥ 1 mo were included in the analysis (57-62). Lines represent the least-squares regression analysis of best linear fit (MINITAB Statistical Software, version 13.32; MiniTab Inc, State College, PA). Regression lines for change (Δ) in plasma phospholipid fatty acids concentrations in g/100 g of fatty acids with ALA doses in g/d are as follows: $\Delta\text{ALA} = -0.60 + 0.12[\text{ALA dose}]$, $r^2 = 0.79$, $P = 0.008$; $\Delta\text{EPA} = 0.34 + 0.06[\text{ALA dose}]$, $r^2 = 0.49$, $P = 0.052$; ΔDHA : no effect of ALA dose on DHA.

EFFECTS OF EPA SUPPLEMENTATION ON PLASMA FATTY ACIDS

Supplementation of adults with ≈ 4 g/d of pure EPA ethyl ester results in significant increases in EPA concentrations in whole plasma and plasma or serum phospholipids, but no increase in



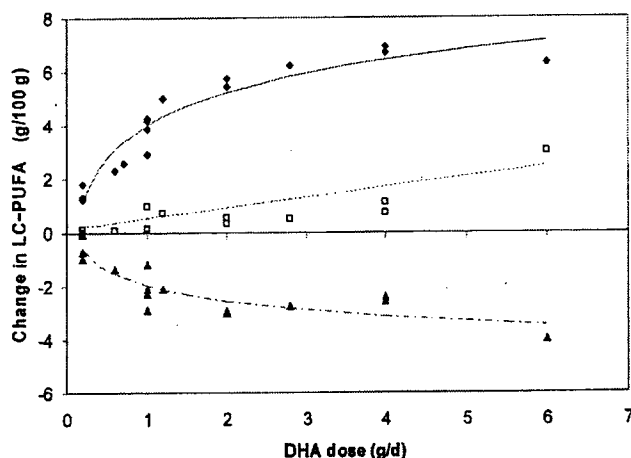


FIGURE 4. Cross-study meta-regression dose-response analyses of the effect of docosahexaenoic acid (DHA) supplementation on plasma phospholipid concentrations of DHA (◆), eicosapentaenoic acid (EPA; □), and arachidonic acid (ARA; ▲). Studies that met the following criteria were included in the analyses: 1) adults ($n \geq 6$ in supplement group) were supplemented daily for 1–6 mo with a DHA source containing little or no EPA [ie, DHA-to-EPA ratio of at least 20:1, included algal DHA triacylglycerol sources (DHASCO or DHASCO-S oils; Martek Biosciences Corporation, Columbia, MD) or pure DHA ethyl esters] and 2) plasma phospholipid fatty acids were reported in g/100 g of fatty acids. Studies involving individuals with metabolic disorders that affect fatty acid metabolism (eg, retinitis pigmentosa or cystic fibrosis) were excluded from the analysis. One study with children was included because the fatty acid results were similar to the results in adults. Published studies identified in PUBMED (National Library of Medicine, Bethesda, MD; 49, 65–70) as well as unpublished data (data on file, Martek Biosciences Corporation) were included in the analysis. DHA supplementation doses ranged between 0.2 and 6 g DHA/d. Changes from baseline fatty acid concentrations for each supplementation group were recorded, and linear (EPA) or logarithmic (DHA and ARA) curve fits were generated. LC-PUFA, long-chain polyunsaturated fatty acids. The equations for the DHA, EPA, and ARA curves, respectively, were as follows: $y = 1.742\ln(x) + 4.023$, $r^2 = 0.91$; $y = 0.150 + 0.389x$, $r^2 = 0.73$; $y = -0.834\ln(x) - 1.977$, $r^2 = 0.77$. Data points were not weighted by sample size of the supplementation group.

DHA concentrations, which is consistent with the poor enzymatic conversion of EPA to DHA (64, 68, 72, 73). A meta-regression dose-response analysis of pure EPA supplementation could not be performed because a sufficient number of studies across a range of EPA doses were not available.

EFFECTS OF SUPPLEMENTATION WITH DHA AND EPA ON PLASMA FATTY ACIDS

Studies with fish oils containing both DHA and EPA have consistently shown increases in both DHA and EPA in plasma (25, 63, 74–77). Blonk et al (74) performed a dose-response analysis of supplementation with marine lipids containing a 2:3 ratio of DHA and EPA at doses up to 6 g total long-chain n-3 fatty acids per day. The results of this study, depicted graphically in Figure 5, suggest a near linear increase in plasma EPA concentrations and an apparent saturable increase in DHA concentrations after supplementation with the combination of fatty acids. The apparent DHA saturation dose was 1.2 g/d, which is considerably lower than when pure DHA is provided and which suggests a possible displacement with EPA in plasma phospholipids. ARA concentrations, as expected, also decreased dose-dependently in response to the combined DHA and EPA supplementation.

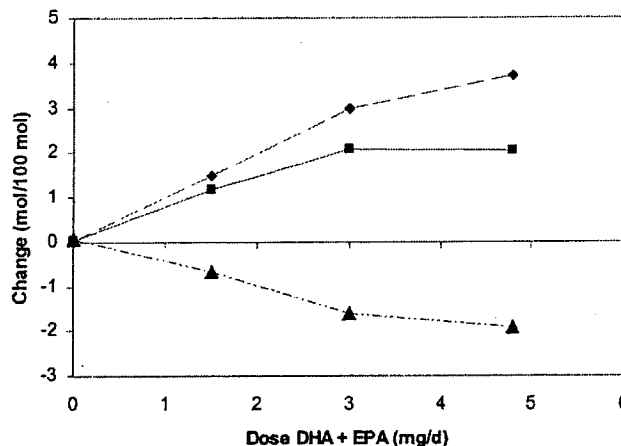


FIGURE 5. Dose-response analysis of the effect of human supplementation with docosahexaenoic acid (DHA) plus eicosapentaenoic acid (EPA) for 12 wk on EPA (◆), DHA (■), and arachidonic acid (▲). Adapted from reference 74 with permission from the American Society for Clinical Nutrition.

COMPARISON OF EFFECTS OF SUPPLEMENTATION WITH ALA, EPA, AND DHA ON PLASMA FATTY ACIDS

The effects of supplementation with similar doses of ALA or pure EPA or DHA ethyl esters on plasma phospholipid fatty acid concentrations are compared across studies in Figure 6. As shown in the figure, both DHA and EPA increase their respective fatty acid in plasma to a similar degree, but ALA is a relatively inefficient source for raising any n-3 fatty acids in plasma phospholipids. EPA and DHA result in similar reductions in plasma phospholipid ARA concentrations. This comparison suggests that the most efficient way to increase plasma concentrations of a particular n-3 fatty acid of interest is to supplement with that specific fatty acid.

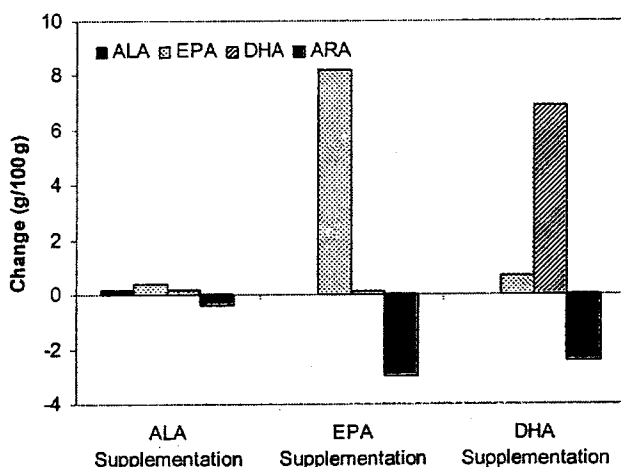


FIGURE 6. Cross-study effects of 3.7 g α -linolenic acid (ALA)/d from canola oil, 4 g pure eicosapentaenoic acid (EPA) ethyl ester, or 4 g pure docosahexaenoic acid (DHA) ethyl ester on the change in plasma phospholipid fatty acid concentrations. ARA, arachidonic acid. Adapted from references 58 and 68 with permission from the American Society for Clinical Nutrition.



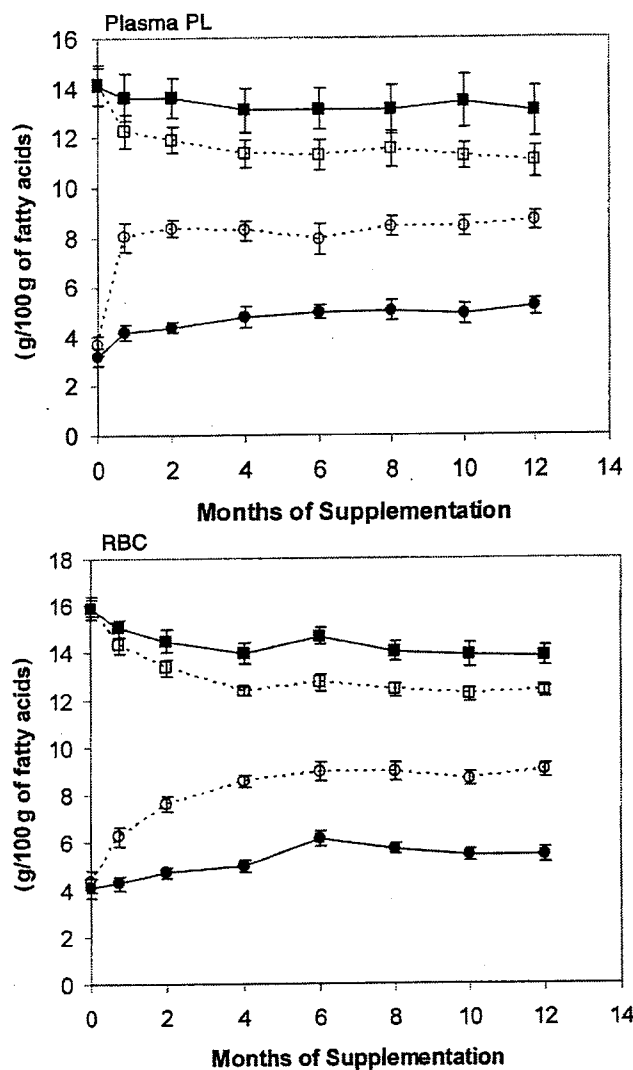


FIGURE 7. Kinetics of docosahexaenoic acid (DHA) supplementation on mean ($\pm$ SEM) plasma phospholipid (PL) and red blood cell (RBC) DHA ($\circ$ and $\bullet$) and arachidonic acid (ARA; $\square$ and $\blacksquare$) concentrations (data on file, Martek Biosciences Corporation, Columbia, MD). Twenty hyperlipidemic adults receiving statin therapy were randomly assigned to receive either 200 mg (solid lines and symbols) or 1000 mg (dashed lines and open symbols) DHA from DHASCO oil daily for 12 mo ($n = 10$ per group). Fasting blood samples were collected at baseline, 3 wk, and 2, 4, 6, 8, 10, and 12 mo and were separated into plasma and RBCs and stored under nitrogen gas at -80°C until analyzed. Plasma lipids were extracted, and phospholipids were isolated by thin-layer chromatography as described (66). RBC total lipids were extracted by the method of Bligh and Dyer (78). Fatty acids from plasma phospholipids and RBC lipids were saponified and methylated with boron trifluoride, and fatty acid methyl esters were identified by gas-liquid chromatography and flame ionization detection as described (66). The protocol was approved by The New England Institutional Review Board (Wellesley, MA).

KINETICS OF LONG-CHAIN $n-3$ SUPPLEMENTATION IN PLASMA AND RED BLOOD CELLS

We have studied the kinetics of DHA supplementation in plasma and red blood cells. As shown in Figure 7, plasma phospholipid concentrations of DHA increased rapidly in a dose-dependent manner after daily supplementation, and, in agreement with the findings of others (79, 80), reached equilibrium

within 1 mo of the start of a new high-dose supplementation regimen. The kinetics of the response was slightly slower with low-dose supplementation. Once new equilibrium concentrations were attained, steady state concentrations were maintained throughout the supplementation period. A similar phenomenon was also reported by Wheaton et al (81) in whole plasma, who showed elevated DHA throughout a 4-y supplementation period. ARA concentrations decreased in a more gradual dose-dependent manner, but eventually resulted in new equilibrium concentrations in plasma phospholipids (Figure 7). Red blood cell kinetics followed a similar pattern; however, it took 4–6 mo after the start of DHA supplementation to reach new steady state concentrations, which is consistent with the slower turnover of these cells. Red blood cell DHA concentrations were maintained thereafter throughout the supplementation period. Other studies with combinations of long-chain $n-3$ supplementation have shown that EPA accumulates more rapidly in plasma and red blood cells than does DHA (75, 79).

The kinetics of DHA and EPA washout after $n-3$ supplementation have been studied by other investigators, who have consistently shown that DHA is more slowly cleared from plasma than is EPA. Marangoni et al (75) reported that whole plasma DHA concentrations decreased slowly once long-chain $n-3$ supplementation had stopped and even after 24 wk were not entirely back to baseline levels. EPA concentrations, on the other hand, decreased rapidly after supplementation was stopped and approached baseline within 4 wk. Others have shown a more rapid washout of EPA and slower reductions in DHA in plasma phospholipids after the completion of fish oil supplementation, and both DHA and EPA decrease more rapidly in plasma cholesterol esters than in phospholipids (76, 82). Katan et al (77) similarly found faster washout of EPA than of DHA from red blood cells.

Differences in the accumulation and retention of DHA and EPA may be related to the lipid moieties in which these fatty acids are stored. DHA is carried predominantly in phospholipids, a more stable lipid fraction in plasma, with lesser portions in triacylglycerol and sterol esters, whereas EPA is more equally distributed between neutral lipids (sterol esters and triacylglycerol) and phospholipids (71, 76, 79). Only small amounts of each of these fatty acids are present in their nonesterified free fatty acid form (80). The differential distribution of DHA and EPA may be linked to differences in the kinetics of washout as well as their saturation dynamics in plasma and availability to tissues.

EFFECTS OF DHA AND EPA SUPPLEMENTATION ON TISSUES

DHA supplementation leads to a dose-dependent increase in plasma phospholipid and red blood cell DHA contents (see Figures 4 and 7). A strong correlation exists between plasma phospholipid and red blood cell DHA contents ($r^2 = 0.72$, $P < 0.001$), and likewise between EPA contents in these 2 compartments in blood ($r^2 = 0.60$, $P < 0.001$) (Figure 8). Others have reported a strong correlation between the combined concentrations of DHA and EPA in red blood cells and plasma (25). Others have also reported a small but significant correlation of cerebral cortex and red blood cell DHA contents in adults, and cheek cell DHA and EPA are correlated with plasma and red blood cell contents of these fatty acids (25, 27). Because plasma and red blood cell contents of DHA and EPA are responsive to dietary intake of

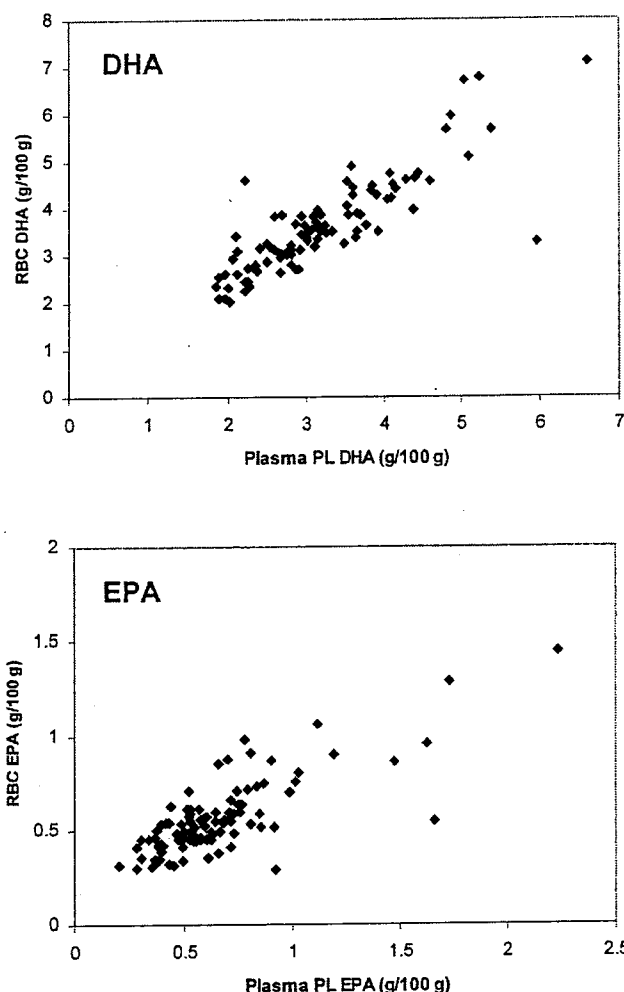


FIGURE 8. Correlation between plasma phospholipid (PL) and red blood cell (RBC) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) concentrations in 96 healthy individuals (data on file, Martek Biosciences Corporation, Columbia, MD). Plasma and RBCs were processed and fatty acids analyzed as described in the legend to Figure 7. The study protocol was approved by The New England Institutional Review Board (Wellesley, MA). Correlation coefficients are as follows: DHA, $r^2 = 0.72$, $P < 0.001$; EPA, $r^2 = 0.60$, $P < 0.001$.

these fatty acids, these correlations suggest plasticity in the fatty acid contents of these organs and that plasma phospholipids or red blood cell DHA contents are generally reasonable markers for tissue DHA concentrations.

Limited data are available on the direct effect of long-chain n-3 supplementation on tissue n-3 contents in humans because of the limited accessibility of human tissues for biopsy. Nonetheless, increases in heart myocardium and skeletal muscle contents of both DHA and EPA in humans have been shown after supplementation with fish oil (23, 25). Others have found increases in adipose tissue and rectal epithelium contents of DHA after 3–6 mo of treatment with DHA (22; J Breslow, personal communication, 2005). Animal studies have also augmented our knowledge about how dietary supplementation with long-chain n-3 fatty acids can affect tissue contents of these fatty acids. Brain, heart, and liver contents of DHA and EPA increased, whereas ARA contents decreased, in a dose-dependent manner in mature rats after 3 mo of supplementation with DHA. Skeletal

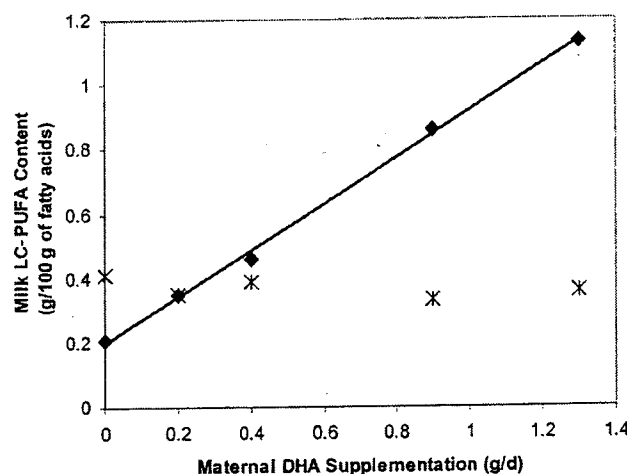


FIGURE 9. Human milk dose response to maternal docosahexaenoic acid (DHA) supplementation. LC-PUFA, long-chain polyunsaturated fatty acids. DHA, ♦; ARA, *. DHA regression line: $r^2 = 0.998$, $P < 0.001$. Adapted from reference 93 with permission from Macmillian Publishers Ltd.

muscle, heart, liver, red blood cell, and bone marrow contents of DHA and EPA increased, with concomitant reductions in ARA, in weanling rats after DHA supplementation for 2 mo (83, 84). Others have shown repletion of brain and retinal DHA after oral supplementation of n-3-deficient animals (85). Together, these studies indicate that in addition to plasma concentrations, tissue concentrations of DHA and EPA can be elevated through dietary supplementation.

MATERNAL SUPPLEMENTATION

DHA availability to the growing fetus and infant is important because this is the period of most rapid brain growth and development. DHA is transferred from mother to the fetus through the placenta and then to the infant postpartum through breast milk. Relative maternal DHA levels decline during pregnancy (86, 87), but maternal plasma DHA concentrations are responsive to DHA supplementation and increase in a dose-dependent manner (88). Infant n-3 concentrations at birth are correlated with maternal n-3 status (89). Infant red blood cell and plasma DHA concentrations after birth are determined largely by diet. DHA contents rapidly decrease by $\approx 50\%$ in plasma phospholipids and red blood cells within 4 mo after birth without an exogenous source of DHA, but are maintained by human milk or DHA-fortified formula feeding (90–92). Human milk DHA content is exquisitely sensitive to maternal diet and increase in a linear, dose-dependent manner (Figure 9) (93). Recent reports of breast milk contents of EPA and DHA in a variety of countries are depicted in Figure 10. DHA contents vary greatly across cultures, primarily reflecting marine food intakes, whereas EPA contents are lower and tend to be less variable. Breast milk DHA contents in American women are among the lowest in the world, generally reflecting the low intake of marine foods in this country.

Infant plasma and red blood cell DHA concentrations are determined by availability of this nutrient from formula or human milk and increase with the dose of DHA in human milk or formula at levels up to $\approx 0.7\%$ of milk formula fatty acids, after

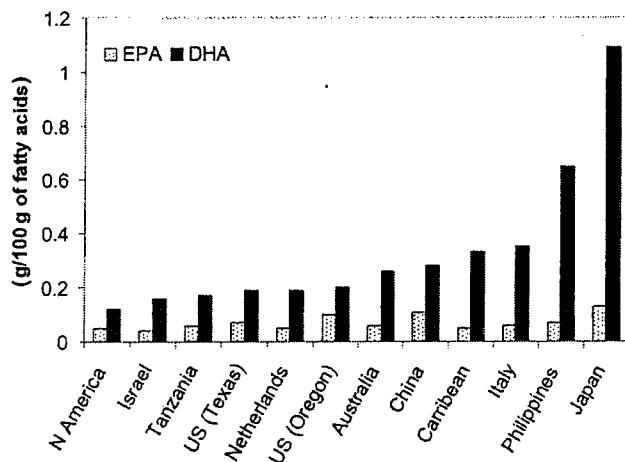


FIGURE 10. Recently published reports of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) contents in human milk in various countries (94–102).

which infant red blood cell DHA concentrations reach near saturation concentrations (103, 104). About 80% of all infant formula sold in the United States now contains DHA, and because infants require a balanced ratio of *n*–6 to *n*–3 fatty acid, these formulas also include ARA. Current US infant formulas contain between 0.1% and 0.35% of fat as DHA. Recent reports suggest that both dose and duration of DHA exposure determine the extent to which infants benefit from the dietary DHA (5, 105).

SUMMARY

Human plasma and tissues are responsive to dietary intake of the long-chain *n*–3 fatty acids, and levels increase in plasma and tissues in a dose-dependent manner. The most effective way to increase a particular *n*–3 fatty acid is to provide that specific dietary fatty acid, because interconversion of the *n*–3 fatty acids is limited in humans. ALA accumulates only to a minor extent, most likely as the result of increased oxidation at higher doses, and modestly raises EPA but not DHA. Plasma phospholipid EPA concentrations increase in a linear manner in response to dietary EPA, whereas dietary DHA causes a dose-dependent, saturable increase in plasma phospholipid DHA concentrations with doses up to ≈ 2 g/d. Both DHA and EPA similarly reduce ARA concentrations in plasma. Tissue contents of long-chain *n*–3 fatty acids increase in response to dietary DHA or EPA. Human milk content of DHA depends on maternal intake of this nutrient, and infant plasma DHA concentrations are responsive to the DHA amounts in their milk or formula feedings. The dose-response information provided herein should be useful in predicting efficient doses of *n*–3 fatty acids for supplementation studies and for developing recommendations for intakes of specific *n*–3 fatty acids.

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A collaborative effort to apply the evidence-based review process to the field of nutrition: challenges, benefits, and lessons learned¹⁻³

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ABSTRACT

Evidence-based systematic reviews evaluating dietary intake and nutritional interventions are becoming common but are relatively few compared with other applications. Concerns remain that systematic reviews of nutrition topics pose several unique challenges. We present a successful collaboration to systematically review the health effects of a common nutrient, n-3 (or omega-3) fatty acids, across a wide range of clinical conditions. More generally, we discuss the challenges faced and the lessons learned during the review, the benefits of systematic review of nutritional topics, and recommendations for conducting and reviewing nutrition-related studies. Through a structured but flexible process, 3 Evidence-based Practice Centers in the Agency for Healthcare Research and Quality program produced 11 reports on a wide range of n-3 fatty acid-related topics. An important resource has been created, through which nutrition and dietetics researchers, clinical dietitians and nutritionists, clinicians, and the general public can understand the state of the science. The process identified challenges and problems in evaluating the health effects of n-3 fatty acid consumption, highlighted challenges to reviewing the human nutrition literature, and yielded recommendations for future research. The goals of these systematic reviews, the processes that were used, the benefits and limitations of the collaboration, and the conclusions of the reviews, including recommendations for future research, are summarized here. *Am J Clin Nutr* 2007;85:1448-56.

KEY WORDS Evidence-based medicine, systematic review, nutrition, dietary supplements, diet, fatty acids, n-3 fatty acids

INTRODUCTION

The process of systematic review has become well established for the evaluation of specific medical treatments and diagnostic tests. The use of this approach for the evaluation of dietary intake and nutritional interventions has become more common recently, but the process has had mixed acceptance by the nutrition and dietetics scientific community because of concerns that nutrition-related topics pose several distinct challenges that are not encountered with systematic reviews of traditional medical interventions. The purposes of this article are to present an example of a large series of systematic reviews of n-3 (or omega-3) fatty acid (FA) consumption and many clinical conditions; to

elaborate on the challenges faced and the lessons learned during this review; and to discuss important caveats about and recommendations for conducting and reviewing nutrition research in general and, more specifically, n-3 FA research. This review does not aim to reproduce conclusions about the effects of n-3 FAs on human health. We also summarize issues related to the collaboration that produced the systematic reviews.

Three centers in the Agency for Healthcare Research and Quality (AHRQ) Evidence-based Practice Center (EPC) program produced 11 evidence reports covering 17 clinical topics and examining the effects of n-3 FAs on clinical outcomes (Table 1). The Office of Dietary Supplements (ODS) and other institutes and centers within the National Institutes of Health (NIH) requested and funded the systematic reviews to summarize the substantial body of evidence on the clinical effects of this particular group of FAs.

Nine reports focused solely on human studies (1-9); 2 either focused on or included questions on animal and in vitro models of arrhythmogenesis or tumor behavior (10, 11). Only the reviews of human studies are discussed here. All reports included

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TABLE 1

Summary of populations, numbers of articles reviewed, interventions, outcomes, and methods used¹

Evidence-based Practice Center and topic	Population	Interventions													Outcomes ³			Synthesis method ⁴	
		Articles ²			Supplement			Diet											
		Citations	Screened ⁵	Included	FO	ALA	Combination ⁶	Fish	Plant	Combination ⁷									
		<i>n</i>									<i>n</i>								
Tufts-New England Medical Center																			
CVD clinical	All adults	7464 ⁸	807	43	5	2		30	5	2	43			148	No	Yes			
CVD risk factors	All adults			123	95	2		20	9	2		123			Yes	Yes			
Organ transplant	Transplant patients of any age	1281	78	30	29					1	21	28	NE	Yes	Yes				
University of Ottawa																			
Eye health	Any age	507	112	16	8			6	1	3	15			1	No	Yes			
Asthma	Any age	1010	159	26	17	3	1	6	1	1	18	7	10	No	Yes	Yes			
Child and maternal health	Mother or child	2049	470	89	60	9		20	5	1	66	44	9	Yes	Yes	Yes			
Mental health	Any age	1212	257	79	25	1		36	2	13	38	56	19	Yes	Yes				
Southern California—RAND																			
Cancer incidence	All adults	5145 ⁹	1228	19	4	4	5	17			19			NE	No	Yes			
Cancer after surgery	GI cancer patients			6			6					6			No	Yes			
Dementia or cognitive decline	Elderly or with PD	5868 ¹⁰	62	5	3	1		4			3	2	NE	No	Yes				
Neurologic diseases	MS; mothers of infants with CP			7	5	2		4			4	3			No	Yes			
Type 2 DM and metabolic syndrome	Adults with type 2 DM or metabolic syndrome	4212 ¹¹	115	32	28	5		1				32	28	No	Yes				
IBD	Adults with IBD			12	10		2					12			Yes	No			
Rheumatoid arthritis	Adults with RA			21	19	1				1		21			Yes	Yes			
Renal disease	Adults with renal disease			8	7		1					8			Yes	Yes			
SLE	Adults with SLE			3	3							3			No	Yes			
Bone density	Adult females			4	3			1	1			4			No	Yes			

¹ AE, adverse events; ALA, plant oil rich in α -linolenic acid or other ALA supplement; CP, cerebral palsy; CVD, cardiovascular disease; DM, diabetes mellitus; Fish, n-3 fatty acids consumed as fish; FO, fish oil (docosahexaenoic acid, eicosapentaenoic acid, or both); GI, gastrointestinal; IBD, inflammatory bowel disease (Crohn disease or ulcerative colitis); MA, meta-analysis; MS, multiple sclerosis; NE, not evaluated; PD, Parkinson disease; Plant, n-3 fatty acids consumed as vegetables, grains, nuts, seeds; Qual, qualitative analysis without meta-analysis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

² The numbers of citations screened, full-text articles screened, and studies included in each report. Combined searches were performed for several topics. The 17 topics are covered in 11 evidence reports (1–11).

³ The number of studies that evaluated each type of outcomes; clinical outcomes; surrogate, intermediate, or risk factor–related outcomes or adverse events. Determination of clinical or intermediate outcomes was made by individual Evidence-based Practice Centers, with the input of technical expert panels and domain experts, depending on the specific topics.

⁴ “Yes” and “No” indicate whether a meta-analysis (MA) was performed for any primary outcomes and whether qualitative (Qual) descriptions alone were performed for any primary outcomes.

⁵ The number of study arms that evaluated each intervention; does not include studies without a specific n-3 fatty acid intervention (eg, association between tissue levels and outcomes).

⁶ Combination of fish oil and α -linolenic acid or of fish and plant sources of n-3 fatty acids.

⁷ n-3 Fatty acids consumed as a combination of foods.

⁸ Combined search for CVD clinical and CVD risk factors.

⁹ Combined search for cancer incidence and cancer after surgery.

¹⁰ Combined search for dementia or cognitive decline and neurologic diseases.

¹¹ Combined search for type 2 diabetes mellitus and metabolic syndrome, IBD, RA, renal disease, SLE, and bone density.

studies that investigated n-3 FA intakes with a range of sources and doses of n-3 fatty acids. The reports focused on the very-long-chain FAs found in fish oils—ie, eicosapentaenoic acid and docosahexaenoic acid—and the n-3 FA found primarily in plant oils—ie, α -linolenic acid (ALA)—in the form of both dietary supplements (eg, encapsulated oils) and food (eg, fish or high-ALA vegetable oils). A structured but flexible systematic review process was used to both maximize consistency across reports and to maintain focus on the questions of interest for each report. The reports can be accessed on the Internet (www.ahrq.gov/clinic/epcquick.htm#Otopics).

SYSTEMATIC REVIEW APPROACH

The primary goal of the systematic reviews was to summarize the evidence to inform the research community of gaps and

limitations in the evidence and to provide a basis for identifying priorities for future research on the effects of n-3 FAs on various clinical conditions, mechanisms of action, and associations between intake and tissue concentrations (eg, serum or platelet phospholipids). The systematic review approach was used to minimize biases by evaluating all the evidence and providing transparency with respect to the method used. The objective and comprehensive approach of a systematic review, in which the data are evaluated with minimal biases about the subject area, is ideally suited for clarifying the nature and strength of the available evidence in controversial areas, such as the health benefits or risks of n-3 FAs. The process allows for conclusions to be drawn on the whole body of evidence rather than on selected evidence and opinions. Systematic reviews, by design, do not attempt to address all possible facets of a topic



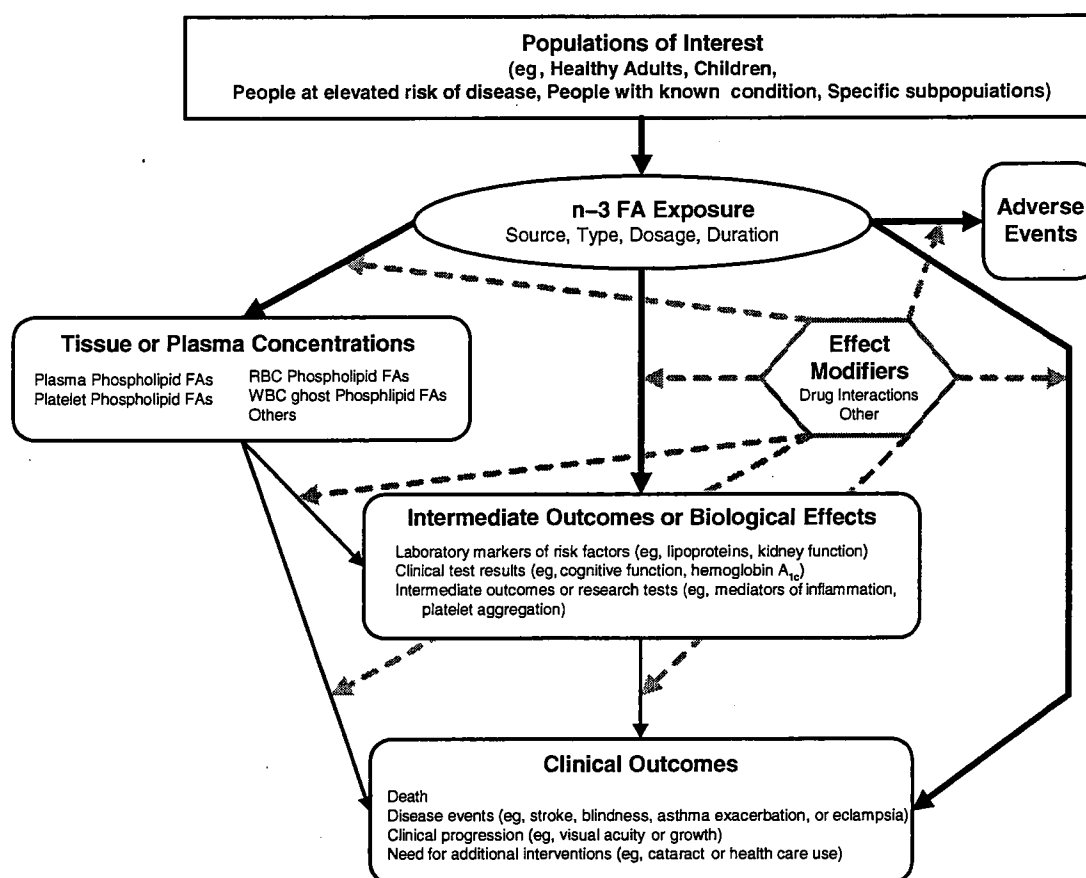


FIGURE 1. Generalized analytic framework of the effects of n-3 fatty acid exposure on outcomes. FAs, fatty acids; RBC, red blood cell; WBC, white blood cell.

but, rather, address focused, answerable, and defined questions. Furthermore, they focus on the types of evidence that best or most completely address the specific questions of interest, and they generally do not include theoretical constructs or anecdotes as evidence of effect.

SYSTEMATIC REVIEW PROCESSES AND METHODOLOGIC COLLABORATION

ODS and other involved parts of NIH identified the primary clinical areas of interest and developed a list of potential key questions. Technical Expert Panels, independent of the EPCs, were convened; they included clinician and researcher domain experts (eg, nutrition scientists, basic scientists, and medical specialists), and representatives from NIH and AHRQ. The technical expert panels played only an advisory and consultative role. Through an iterative process, together with the technical experts, each EPC explicitly refined the key questions for each clinical outcome by defining the relevant populations, interventions, comparators, outcomes, and study designs of interest for that outcome (12). The process provided sufficient flexibility to ensure that the reviews addressed the questions of greatest interest to the research community and funding agencies and also accommodated differences in the state of science across the wide range of clinical outcomes.

The reports included common approaches, when feasible, to ensure consistency in capturing n-3 exposure information, in

conducting literature searches, and in the use of methodologic quality ratings. However, to accommodate differences across the wide range of clinical outcomes of interest and differences in the needs and interests of sponsoring NIH institutes and centers, the reports exhibited flexibility through variations in the framework questions, eligibility criteria for study designs, populations, and types of outcomes.

The EPCs collaborated on several methodologic elements, including centralized literature searching, common approaches to assessing and reporting the quality and applicability of the evidence, standardized evidence tables, and the use of what we called "summary matrixes" to summarize the overall quality and applicability of the literature. We also sought common a priori definitions of variables, including interventions, and, where appropriate, common constructs within analytic frameworks. For example, we agreed on allowable sources of n-3 FAs, excluding fried fish. However, each EPC supplemented the electronic literature searches and independently developed specific screening criteria for each topic. Cognizant of the need to review the breadth of evidence and of the fact that including only randomized trials would have been insufficient for reviews of diet and chronic diseases (13), we generally included both randomized trials and observational studies.

We developed a common approach to formulating analytic frameworks for each report (Figure 1), which were based on the US Preventive Service Task Force recommendation (14). Analytic frameworks provide several advantages to both the

TABLE 2

Summary of body of evidence and overall results for fish oils and fish¹

Topic	Quality ²																				Overall results		
	Study design ²					Intervention duration		Grade			Jadad score					Applicability ⁴							
	RCT	nRCT	CC	PrC	R/XS	Minimum	Maximum	Good	Fair	Poor	5	4	3	2	1	0	Broad	Moderate	Narrow	Clinical	Intermediate		
	<i>n</i>							<i>n</i>									<i>n</i>						
CVD clinical	6		4	25		1.5 y	30 y	15	15	5	1	2	2		1		1	30	4	Benefit			
CVD risk factors	107			3	3	4 wk	3.5 y	16	72	19	10	26	38	26	7		13	72	28		No benefit ⁵		
Organ transplant	22	2		5		1 d	8.7 y	21	8	1	12	6	3				NE			Inconclusive	Inconclusive		
Eye health	2		2	3	8	3 wk	12 y	3	5	7		1		1			7	3	3	Inconclusive			
Asthma	10	4	1	1	7	10 d	10 y	9	8	6	1	3	3	3			3	1	15	Inconclusive	Inconclusive		
Child and maternal health	55		3	6	15	3 wk	4 y	22	28	26	16	4	12	19	4		48	19	5	Inconclusive	Inconclusive		
Mental health	23	1	5	6	38	4 wk	9 y	12	34	26	3	7	10	3			38	18	15	Inconclusive	Inconclusive		
Cancer incidence				19		2 y	30 y	2	14	3							6	13		No benefit			
Cancer after surgery	6					5 d	8 wk	3	2	1	2	1		1	1		5		1		Mixed		
Dementia or cognitive decline	1			4		1 y	7 y		3	1						1		4	1	Inconclusive ⁶	Inconclusive		
Neurologic diseases	1	2	2	1		2 y	24 y		1	2				1				6		Inconclusive			
Type 2 DM and metabolic syndrome	29			1		2 wk	1 y		NE			4	8	13	4		1	11	16		Mixed		
IBD	12					3 mo	2 y		NE		4		3	5			2		8		Mixed		
Rheumatoid arthritis	19			1		10 d	15 mo		NE			5	8	3	3			7	9		Mixed		
Renal disease	8					2 mo	5 y		NE			3	2	3			1	5	1		Inconclusive		
SLE	3					3 mo	1 y		NE		1		2				1	2			Inconclusive		
Bone density	4			1		1 y	18 mo		NE					3	1		1		3		Inconclusive		

¹ CC, case-control study; CVD, cardiovascular disease; DM, diabetes mellitus; IBD, inflammatory bowel disease; NE, not evaluated; nRCT, nonrandomized controlled trial; PrC, prospective cohort study (no control); R/XS, retrospective or cross-sectional study; RCT, randomized controlled trial; SLE, systemic lupus erythematosus.

² The number of studies that were RCTs, nRCTs, CCs, PrCs and R/XSs.

³ The number of studies with each rating. RCTs were also scored on the Jadad scale, for which 5 is the maximum score. Studies not accounted for in the table were not evaluated for quality.

⁴ The number of studies with each rating. Studies not accounted for in the table were not evaluated for applicability.

⁵ No overall benefit; however, improvements in hypertriglyceridemia and small reductions in blood pressure were found. The effects found were considered unlikely to have a clinically significant effect on CVD.

⁶ Evidence was found of a possible reduction in incidence of non-Alzheimer dementia with fish consumption.

reviewers and the readers of systematic reviews by providing a visual map outlining specific linkages among the populations of interest, exposures, modifying factors, and outcomes of interest. Specifically, these frameworks depict the chain of logic that evidence must support to link the exposure (eg, consumption of n-3 FAs) to clinical outcomes. This process helps guide the development of key questions, determine study eligibility criteria, and interpret and contextualize relevant studies.

Although it is generally accepted that deficiencies in study design, conduct, analysis, and reporting may lead to biased results, there is little agreement on whether any specific study factor consistently biases or restricts the applicability of given studies (15–19). All 13 AHRQ-sponsored EPCs use a similar set of basic criteria to assess study quality; however, no uniform approach is used to determine which specific criteria are used. For the purposes of this simultaneously generated set of reports, we agreed a priori to standardize our approaches to allow better comparisons across reports. Thus, for all reports, the validity of included randomized trials was assessed by using the Jadad score, which assesses randomization, double blinding, and drop-outs and withdrawals on a 5-point scale (20); adequacy of allocation concealment as described by Schulz et al (18); and a 3-category system (good, fair, or poor) that qualitatively grades the likelihood of bias based on a range of potential quality factors. The 3 EPCs did not, however, use the same approaches for grading nonrandomized studies: some used the 3-category system and some used other scales (21). In addition, given the unique nature of the data in some reports, topic-specific quality scales were used. Each study was also given an applicability score

(broad, moderate, or narrow) to summarize its relevance to the populations of interest for each report question.

Among the strengths of coordinating a large number of reviews across several EPCs was that we could achieve consistency in several centralized processes for core components of the reviews. However, because each EPC has its own systems and methodologic approaches, we were unable to achieve complete consensus regarding data analysis (eg, evaluation of net changes or of final values), types of outcomes of interest (eg, definition of clinical or intermediate outcomes), or style of reporting (eg, exact format of tables).

OVERVIEW OF EVIDENCE-BASED REPORT FINDINGS

The primary clinical conditions reviewed by the 3 EPCs are summarized in Table 1, Table 2, and Table 3. The specific questions varied by topic, but they were intended to cover the effects of n-3 FA consumption either on prevention or treatment of specific disease states or on the risk factors or markers of the diseases.

When the included studies are grouped by topic, the largest groups were studies of cardiovascular disease (CVD)-related endpoints (32%), of child and maternal health (17%), and of mental health (15%) (Table 1). Most interventions evaluated the effect of fish-oil supplementation (57%) or fish diets (26%) (Tables 1 and 2). The reports summarized the evidence of adverse events and of any differential effects among populations or risk groups.



TABLE 3

Summary of body of evidence and overall results for α -linolenic acid (ALA) (supplements and plant-source diet)¹

Topic	Quality ³																			Overall results	
	Study design ²					Intervention duration		Grade			Jadad score				Applicability ⁴						
	RCT	nRCT	CC	PrC	R/XS	Minimum	Maximum	Good	Fair	Poor	5	4	3	2	1	Broad	Moderate	Narrow	Clinical	Intermediate	
	n																				
CVD clinical	5			3	1	1 y	10.5 y	2	6	1			2	3		2	5	2	Inconclusive		
CVD risk factors	11			1	1	4 wk	2 y	1	2	8			1	2	4	4	3	6	4	Inconclusive or no benefit	
Organ transplant				1		ND				1							NE			Inconclusive	
Eye health					2	2 mo	4 y		2								2			Inconclusive	
Asthma	2				2	2 wk	18 mo	1	2	1			1	1		4				Inconclusive	
Child and maternal health	9		1	1	3	1 wk	52 wk	3	6	5	1		3	3	2	8	3	1		Inconclusive	
Mental health	1		5	2	8	1 mo	2 y	1	9	6				1		7	5	4		Inconclusive	
Cancer incidence				5		2 y	15 y	3	1	1						1	4			Inconclusive	
Cancer after surgery																					
Dementia or cognitive decline				1			3.9 y	1										1		Inconclusive	
Neurologic disease				2			14 y ⁵	1	1								2			Inconclusive	
Type 2 DM and metabolic syndrome	5					4 wk	3 mo		NE					2	3			2		Inconclusive	
IBD																					
Rheumatoid arthritis	1						3 mo		NE					1						Inconclusive	
Renal disease																					
SLE																					
Bone density																					

¹ CC, case-control study; CVD, cardiovascular disease; DM, diabetes mellitus; IBD, inflammatory bowel disease; ND, no data (or unclear data); NE, not evaluated; nRCT, nonrandomized controlled trial; PrC, prospective cohort study (no control); R/XS, retrospective or cross-sectional study; RCT, randomized controlled trial; SLE, systemic lupus erythematosus.

² The number of studies that were RCTs, nRCTs, CCs, PrCs, and R/XSs.

³ The number of studies with each rating. RCTs were also scored on the Jadad scales, for which 5 is the maximum score. Studies not accounted for in the table were not evaluated for quality.

⁴ The number of studies with each rating. Studies not accounted for in the table were not evaluated for applicability.

⁵ Duration not reported for 1 study.

The reports included studies documenting dietary intakes of n-3 FAs from both marine and plant sources, as either nutritional supplements or dietary components. Approximately two-thirds of the study arms evaluated n-3 FA supplements; the remaining study arms evaluated food sources (eg, plant oil spreads or fish). Fish oils constituted the source of n-3 FAs in more than half of the studies, and fish did so in approximately one-quarter of the studies. Only 10% of interventions provided ALA alone (without fish oils) (Tables 1 and 3); thus, for approximately one-third of the clinical conditions examined, no studies evaluated ALA. The types, designs, and durations of reviewed studies varied widely across reports.

In part, this variability reflects the intensity with which each clinical area has been researched and the variations in eligibility criteria used by the different reports. For most reports, randomized controlled trials were the studies most commonly reviewed, although prospective and retrospective observational studies also were commonly included. Studies of fish and fish oil were more commonly randomized controlled trials than studies of ALA. Overall, approximately one-quarter of the studies were deemed to be of good quality, and one-quarter were of poor quality. Among the randomized trials, only about one-quarter had a 4 or 5 (out of 5) Jadad score. Similarly, approximately one-quarter of the studies were of broad applicability, and one-quarter were of limited applicability. The overall quality and applicability of studies varied substantially across clinical topics. Fish-oil studies were similar to ALA studies in their overall methodologic quality and applicability, except that few ALA randomized trials received high Jadad scores.

For most topics reviewed, the evidence of health benefits of either fish oils or ALA was inconclusive. The paucity of studies,

particularly of randomized trials, and the heterogeneity of interventions evaluated (ie, the wide variety of FA doses, combinations, forms tested, and outcomes measured) prevented firm conclusions. Many studies were small, methodologically flawed, and of limited applicability, and they frequently were heterogeneous in their results. However, incompletely reported data, failure to fully describe study designs or conditions, and differences among participant populations often made it difficult to meaningfully explain the heterogeneity in findings.

The evidence was adequate to attribute a health benefit to consumption of supplemental fish oil or dietary fish only with respect to a reduction in cardiovascular events, principally as secondary prevention. In certain persons and at various doses, fish oils reduce serum triacylglycerol concentrations, reduce blood pressure by a small but significant amount (≈ 2 mm Hg), reduce corticosteroid use in patients with rheumatoid arthritis, and reduce the risk of non-Alzheimer dementia. No association was found between n-3 FA consumption and cancer incidence, although, for most types of cancer, the data were not sufficient to exclude possible associations.

Most of the evidence of adverse events was found in the studies of CVD-related outcomes. Even among these studies, however, only 148 of 395 (37%) evaluated articles provided any information on adverse events, and the reporting of adverse events was generally inadequate and incomplete. In particular, rates of adverse events were not systematically or consistently monitored and reported. Overall, given these caveats, no evidence of substantial adverse events related to n-3 FA consumption beyond mild gastrointestinal symptoms was found. However, the lack of protocols for monitoring adverse events could have resulted in underreporting or biased reporting of actual effects.



ADDED VALUE OF SYSTEMATIC REVIEW REPORTS

The dissemination and added value of these reports have already been observed. Since their release, several national and interagency meetings have specifically used these reports to guide research agendas. ODS and the National Heart, Lung, and Blood Institute gathered domain experts in a workshop to help the NIH determine the future research on n-3 FAs and CVD that should be funded and the general design that should be used for those studies. By integrating information from the evidence reports with the participants' expertise, the workshop concluded that the need for further studies of the effects on established cardiovascular risk factors (eg, lipoproteins and blood pressure) is limited, but that a well-designed, long-term randomized trial of both fish-oil and ALA supplements should be conducted to assess their effects in a high-risk population (22).

Similarly, the National Institute of Environmental Health Sciences, the Office of Rare Diseases, and ODS convened a workshop to evaluate the possible development of a prevention trial designed to assess the effect of n-3 FA supplementation on asthma and allergy during pregnancy and infancy. Findings from these reports have also been presented to researchers at other NIH-sponsored meetings and at meetings such as Experimental Biology, Translating Research into Practice and Policy, and SupplySide East, where the goals of the presentation were to highlight the issues related to the value of the current evidence and to offer suggestions to improve future research. A larger audience has also been reached through journal publications, press releases, and the general press (23-33).

LIMITATIONS OF THE EVIDENCE AND RECOMMENDATIONS FOR FUTURE RESEARCH

Many deficiencies that are common in the current medical and health literature were also present in this field, as shown in **Table 4**. For example, inadequate reporting of randomized trials can introduce bias into effect estimates of interventions and clearly makes interpretation of studies and their possible biases problematic (34, 35). Other deficiencies were more specific to both n-3 FA research and nutrition research in general (Table 4).

Considerable energy has been devoted to providing guidance to authors in improving their reporting of randomized trials, such as the CONSORT Statement (35), which is associated with better reporting of randomized trials (36). Thus, to improve the utility of publications, the CONSORT criteria should be used as a guideline for reporting all future trials. The CONSORT Statement has recently been extended to help with reporting trials of herbal interventions (37). Whereas many of the statement's considerations may apply to trials of nutritional supplements and diets, further refinements to the criteria may be needed for this field of study.

Additional future research recommendations include improving and developing plausible models of disease interactions in animal and in vitro models, and ensuring that hypotheses are tested for both primary and secondary prevention (ie, in persons with and without a history of the conditions of interest). For several clinical conditions, such as dementia and cancer, dietary assessments starting at a relatively young age with very long-term follow-up are necessary to evaluate primary prevention. Such designs could feasibly be implemented by converting randomized trials of dietary or supplement interventions into long-term observational studies.

TABLE 4

Common limitations of current studies of n-3 fatty acids

Limitations common to health sciences research
Inadequate study design (eg, no control group)
Inadequate analysis (eg, no direct comparison between interventions)
Poor reporting of studies (eg, missing data; inconsistencies between abstract, text, and tables)
Inadequate descriptions of the interventions, controls, and outcomes
Use of intermediate outcomes that either have not been shown to be associated with condition of interest, are not in standard use, or have not been validated
Small study size, which limits the ability to interpret "negative" findings
Inclusion of only highly narrow populations, which limits applicability
Short-term follow-up and lack of multiple testing for progression
Failure in crossover studies to adequately analyze or report results, including
No accounting for the need for a washout period
No reporting of outcomes at all stages of the study
Improper analytic techniques
No analysis of the comparison between intervention and control arms of the study
Inadequate reporting of safety data
Limitations specific to n-3 fatty acid or other nutrition research
Large variety of dietary components (ie, 3 different n-3 fatty acids), doses, and sources (eg, different fish or oil sources) greatly complicate determinations of the effects of the class of nutrient (n-3 fatty acids)
Lack of evaluation or analysis of potential confounders of nutrient-outcome relations (eg, consumption of other fats, antioxidant vitamins, or prooxidant nutrients; changes in weight; and other risk factors for disease outcomes)
Possible effect of other bioactive food components (eg, sterols or mercury) consumed with the n-3 fatty acids were generally not considered
Failure to describe baseline consumption of n-3 fatty acids or of background diet
Failure to evaluate changes in ratio of n-6 to n-3 fatty acid consumption
Failure to evaluate changes in ratios of eicosapentaenoic acid to docosahexaenoic acid and consumption of α -linolenic acid
Failure to use validated measures of dietary intake or biomarkers of n-3 fatty acid exposure
Estimation of n-3 fatty acid exposure only from single measures of dietary consumption
Failure to validate n-3 fatty acid consumption with tissue concentrations
Few direct comparisons of different sources, doses, and ratios of fatty acids
Inadequate information on treatment products (eg, purity, composition, presence of other bioactive compounds, or effective masking of taste or smell characteristics)
Inadequate description and characteristics of placebo products and carrier substances

The applicability and strength of studies would be increased if research groups begin to collaborate on multicenter trials. Such study designs could also stretch scarce research dollars and simultaneously allow consideration of confounding factors. Furthermore, comprehensive and systematic adverse event-monitoring systems should be developed and incorporated into future studies. Many other specific recommendations that are also applicable to nutrition research in general were made. Notably, one EPC made the practical suggestion to piggyback the assessment of different sets of clinical outcomes (eg, those for mental health) onto studies primarily designed to evaluate other sets of clinical outcomes (7).



DISCUSSION

The series of evidence reports on n-3 FAs commissioned by AHRQ has provided an important resource for nutrition and dietetics researchers, clinical dietitians and nutritionists, clinicians, and consumers with respect to what is known—and not known—about the effects of increased consumption of n-3 FAs. They have also helped NIH set research agendas and priorities. This review elaborates on the benefits of the reports, lessons learned during the systematic review process, and recommendations for conducting and reviewing nutrition research.

These reports highlight an important message to the nutrition research community that, whereas future research on many clinical questions is necessary, our knowledge of the health effects of n-3 FAs (and by extension, other nutrients) will not significantly progress simply through the conduct of more studies. Advances in knowledge will require substantial improvements in the quality of study rationale, design, conduct, and documentation. Additional studies done without significant methodologic improvements will be unlikely to meaningfully improve the state of the science with respect to the health effects of nutrients.

For practitioners and consumers, whereas the reviews provide few definitive statements about known effects of n-3 FA consumption, they do provide a resource to help understand the evidence. They provide a comprehensive and rigorous evaluation of the literature that would be difficult for individuals to replicate. There was frequently a lack of sufficient information indicating that a particular intervention is effective, but the reviews provide a basis for fully informed decisions regarding n-3 FA consumption.

The reports highlighted important differences between fish oils and the plant oil ALA (eg, several benefits of fish oils for CVD and risk factors have not been found with ALA). They have also highlighted several important deficiencies in the research findings currently available. Despite a large evidence base, few clinical areas exist in which it is possible to make adequate assessments of the possible benefit or harm of n-3 FAs. This situation is largely a result of the small number of well-designed, high-quality studies—including both randomized controlled trials and observational studies—that have been conducted. Even fewer of these studies clearly defined interventions or adequately controlled for factors that are important in most nutrition-related studies, such as background diet and equivalent control subjects.

Interpreting human studies in the area of nutrition can be considerably more challenging than is interpreting drug trials. One factor is energy balance. To avoid changes in body weight that can confound the outcome measures (eg, LDL cholesterol), if a food is added to or subtracted from the diet, another food must be subtracted from or added to the diet to compensate. Thus, it is necessary to use controls that are energetically equivalent. Furthermore, the effects of increased consumption of a food product (eg, fish or fish-oil supplements) may differ depending on whether a person's background diet is already rich in that food (eg, fish). In observational studies, if a person reports eating a diet high in fish, he or she is likely to be eating less of another food, perhaps meat. Among observational studies, it is critical that potential confounding factors—such as background diet, food preparation method (eg, frying), weight, health status, and disease-specific risk factors—be accounted for (38). In addition, nutrients such as n-3 FAs are derived from sources that may contain other bioactive compounds that may affect the clinical

outcomes of interest. The presence of these compounds may confound the effects of the nutrient of interest, particularly if these other compounds are not accounted for in the control interventions. It is also important to address the balance between different dietary fats (eg, n-3 and n-6 FAs; eicosapentaenoic acid, docosahexaenoic acid, and ALA) or altered intakes of other FAs (eg, saturated fatty acids). Diets or even nutritional supplements contain many nutrients that may interact, which adds a layer of complexity to their evaluation (39). In our review of the literature, it became apparent that only rarely were these factors taken into consideration.

Whereas, overall, better research and reporting are needed, the systematic reviews highlighted the possibility that adequate evidence for the effects of fish oils on a few outcomes, including improvements in lipoproteins, triacylglycerols, and blood pressure, may exist from which to base recommendations. Acknowledgment that these effects of n-3 FAs have been adequately studied could allow scarce resources to be better spent in evaluating underresearched areas.

Our evidence reports showed that evidence-based methods can be successfully applied to the evaluation of nutrition topics. These reports have highlighted the numerous clinical topics for which the evidence is inconclusive and on which further high-quality studies are needed. They also can provide the basis for planning future research. Our experience also serves to show the resource- and time-intensive nature of the systematic review process. The coordination among the EPCs allowed certain efficiencies and provided an excellent opportunity for each group to learn new approaches and improve its own processes. However, once data review began, coordination of each step was difficult to implement. Nevertheless, all groups found that conscientiously using a rigorous approach to define review parameters was essential to bringing focus to the clinically important questions being examined. Through consensus and collaboration, we ensured that the review questions covered the areas of interest but were clear and concise, which led to more focused reviews and made all aspects of the process, such as the searches, more efficient. The collaboration among the 3 EPCs, AHRQ, NIH, and the technical experts and the large scope of the overall project maintained a broad perspective precisely to avoid potential pitfalls of other systematic reviews of this topic (39–43).

The resource-intensive nature of these systematic reviews also raises practical questions. The benefits of an overarching set of reviews continually need to be balanced with the costs and delays inherent in such a large endeavor (almost 29 000 citations and 3300 full articles were screened, and 523 studies were reviewed). However, the breadth of these reviews should allow future updates to be sufficiently focused, both by clinical topic and on particular key questions, so that they can be completed relatively quickly. As time passes, we expect an increasing need for updates to these reports. Systematic reviews are most useful when they are kept current, because evidence is continually evolving as new research becomes available. Health care interventions that are currently accepted as effective may in the future be shown to be ineffective or harmful, or vice versa. Ignoring the emergence of new information could undermine the validity of even the most current, high-quality systematic reviews. As the number of newly published studies grows, depending on their findings, the current systematic reviews may become less authoritative.



The systematic review process, particularly with the coordination of the 3 EPCs, was the definitive approach to understanding the state of the literature on n-3 FAs and human health. These systematic reviews were able to confirm several beneficial health effects of increased n-3 FA consumption but they also found major deficiencies in the literature and highlighted the clinical areas, including adverse effects, in which the evidence is inadequate. The wide-ranging group of researchers involved in the reviews, both collectively and individually, capitalized on their experience to influence the thinking, both at NIH and among nutrition researchers, about the future research that is needed to address the gaps in the evidence, rather than to add further inadequate data to the literature.

The authors' responsibilities were as follows—all authors: conception of the collaboration; EMB, TAH, SJN, and AHL: data acquisition and the draft of the manuscript; and all authors: critical revision of the manuscript. None of the authors had a personal or financial conflict of interest.

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Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*

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Abstract

The ascomycetous yeast *Yarrowia lipolytica* (formerly *Candida*, *Endomycopsis*, or *Saccharomyces lipolytica*) is one of the more intensively studied 'non-conventional' yeast species. This yeast is quite different from the well-studied yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* with respect to its phylogenetic evolution, physiology, genetics, and molecular biology. However, *Y. lipolytica* is not only of interest for fundamental research, but also for biotechnological applications. It secretes several metabolites in large amounts (i.e. organic acids, extracellular proteins) and the tools are available for overproduction and secretion of foreign proteins. This review presents a comprehensive overview on the available data on physiology, cell biology, molecular biology and genetics of *Y. lipolytica*.

Keywords: *Yarrowia lipolytica*; Physiology; Cell biology; Cell cycle; Genetics

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1. Introduction

In the last two decades the group of so called 'non-conventional' yeasts received more and more attention in fundamental research and biotechnology. The term 'non-conventional' was originally used to differentiate this group of yeasts from the more commonly used —'conventional'— and already well studied yeasts *Saccharomyces cerevisiae* and, with some restrictions, *Schizosaccharomyces pombe*. Nowadays about ten species among these non-conventional yeasts have been investigated more intensively with respect to their physiology, genetics, molecular biology, and biotechnical application. A summary of these data and the special tools developed has been published recently [1].

The ascomycetous yeast *Yarrowia lipolytica* (originally classified as *Candida lipolytica*) is one of the more intensively studied 'non-conventional' species. With the emergence of single-cell protein (SCP) projects in the mid-sixties, a strong industrial interest arose from the fact that strains of this species were able to use *n*-paraffins (which were cheap and abundant in this period) as sole carbon source. It was also observed that *Y. lipolytica* was able to produce high amounts of organic acids (2-ketoglutaric acid and

citric acid) when grown on these substrates [2]. Large-scale industrial production of citric acid or of SCP using *Y. lipolytica* thus permitted the accumulation of extensive data on its behavior in very large fermentors.

Three reviews have been already published which describe the historical development of this research and summarize main data on physiology, genetics, molecular biology, and biotechnological application of this fungus [3–5].

This review will give a comprehensive overview on the physiology and genetics of this yeast. It will also show recent areas of interest which include both fundamental and applied topics.

2. Natural habitats, taxonomy, and phylogenetic relationship

Y. lipolytica strains are readily isolated from dairy products, but also from shoyu or from salads containing meat or shrimps. The inability of this yeast to survive under anaerobic conditions permits their easy elimination from dairy products, contrary to *Kluyveromyces marxianus* strains for example [6]. An extensive description of assimilated substrates is done

by Kreger-van Rij [7] and Barnett et al. [8]. Glucose, alcohols, acetate, and hydrophobic substrates like fatty acids or alkanes, but not sucrose are used as carbon sources.

The species was originally classified as a *Candida*, since no sexual state had been described. The perfect form of *C. lipolytica* was identified in the late sixties by Wickerham at the Northern Regional Research Laboratory of the USDA at Peoria. A culture isolated in 1945 from a jar of fiber tailings in a corn processing plant was found to form asci attached to hyphal elements when put on suitable media. One to four spores of various size and shape could be isolated from these asci, but spore viability was found to be very low. Two mating types, called A and B, were identified among the progeny. Nearly all other wild type isolates from the species would mate to one of these two types, albeit at very low frequency, suggesting that most natural isolates are haploid (or near haploid). The perfect form was reclassified first as *Endomycopsis lipolytica* [9], then as *Saccharomycopsis lipolytica* [10], and finally as *Yarrowia lipolytica*.

Y. lipolytica is not considered as pathogenic [11].

Several observations suggested that *Y. lipolytica* may have diverged considerably from other ascomycetous yeasts: high GC content, unusual structure of rDNA genes coupled with a lack of RNA polymerase I consensus sequences found in other yeasts [12], higher eukaryotic-like size of snRNA [13], and of 7S RNA [14]. Homologous genes tend to display a low level of similarity (typically in the 50–60% range at amino acid level) with their counterparts in *S. cerevisiae*, *K. lactis* or *C. albicans*, thus hindering in most cases attempts to clone homologous genes by means of Southern hybridization. Naumova et al. [15] showed that *Y. lipolytica* genes did not hybridize detectably with DNA from species of the genera *Saccharomycopsis*, *Endomyces* or *Endomycopsella*, some of which were formerly classified in the same genus as *Y. lipolytica*. Similarly, only few genes of *Y. lipolytica* seem to be directly expressed in *S. cerevisiae*, suggesting that RNA polymerase II promoters and/or associated transcriptional factors have diverged considerably. Recent data on 7S RNA genes, on intron structure, or on *ARS* function confirmed that this species was quite peculiar when compared to most other yeasts. Evolutionary trees based on se-

quence comparison of genes encoding well conserved functions (glycolytic genes, ribosomal RNA genes) locate *Y. lipolytica* on an isolated branch, clearly separated from *S. pombe* on one hand, and from the bulk of other ascomycetous yeasts on the other [16,17]. A truly positive aspect of this divergence is that the observation of structural conservation between *Y. lipolytica* and other yeast genes is much more likely to reflect functional constraints than when it happens between closely related yeasts.

3. Physiology

3.1. Assimilation of carbon sources

3.1.1. Utilization of hydrocarbons as carbon source

Y. lipolytica can use *n*-alkanes and 1-alkenes as carbon sources [18–21]. Polymethylated and chlorinated alkanes are also assimilated by this fungus [22,23]. However, only few enzymes are characterized and no gene involved in the early steps of degradation of these compounds has so far been cloned.

The first step in the assimilation is likely to be an emulsification at the cell surface to form small droplets which can be internalized. A 27 kDa extracellular emulsifier, called liposan, is induced in cells growing on *n*-alkanes [24,25]. Liposan contains 88% carbohydrate and 12% protein. After entry into the cell, *n*-alkanes are hydroxylated by a cytochrome P-450 monooxygenase system. Cytochrome P-450 was detected in several strains of *Y. lipolytica* [26–28]. The synthesis of this enzyme is induced during growth on *n*-alkanes, but not on glucose, ethanol or acetate. Differences in sensitivity to glucose repression during growth on hexadecane and decane indicate the presence of different regulated cytochrome P-450 genes in *Y. lipolytica* [29]. Cytochrome P-450 is localized in the membrane of the endoplasmic reticulum as shown by subcellular fractionation of alkane-grown cells of *Y. lipolytica* [29]. 1-alkanol formed after the first step is further oxidized by a membrane-bound fatty alcohol oxidase [30,31], not by NAD(P)-dependent alcohol dehydrogenases, which are also present in *Y. lipolytica* [32].

Several alkane-non-utilizing mutants have been isolated and characterized [33–36]. Uptake of *n*-alkanes is inducible and due to active transport. Muta-

tions in 16 loci caused a significant reduction in *n*-alkane uptake [34]. Mauersberger [35] characterized three subtypes of *alkA* mutants, which were unable to utilize either all types of *n*-alkanes (*alkAa*), or only short ones in the C₈ to C₁₂ range (*alkAb*), or only long alkanes in the C₁₆ range (*alkAc*). These data indicate that *Y. lipolytica* contains either several length specific alkane-uptake systems or specific cytochrome P-450 monooxygenases.

3.1.2. Fatty acids biosynthesis and degradation

Fatty acid biosynthesis and degradation were initially investigated by Kamiryo et al. [37] and a few reports were issued on the use of *Y. lipolytica* for the stereospecific conversion of hydroxylated long chain fatty acids into γ -lactones [38].

Kohlwein and Paltauf [39] detected at least two fatty acid carrier systems in *Y. lipolytica*, one being specific for C₁₂ or C₁₄ fatty acids, the other for C₁₆ and C₁₈ saturated or unsaturated fatty acids. Octanoic acid and decanoic acid are not taken up by this yeast. Contrary to *S. cerevisiae*, an intracellular 100 kDa fatty acid binding protein is readily induced in *Y. lipolytica* when grown on palmitate [40]. A low molecular mass fatty acid binding protein (15 kDa) has been isolated from the cytosol of this yeast recently [41].

An anabolic acyl-CoA synthetase I activity was found in peroxisomes, mitochondria and cytoplasm and was shown to be required for the incorporation of exogenous fatty acids into cellular lipids. Mutants deficient in this activity have been isolated [37].

The gene *FAS1*, encoding the pentafunctional β -subunit of the fatty acid synthetase was cloned and sequenced [42,43]. Its overall structure was very similar to that of *S. cerevisiae*.

A catabolic acyl-CoA synthetase II was found exclusively in peroxisomes, and was required for the β -oxidation of fatty acids. A mutant initially reported as affecting this activity was later shown to affect another step (Kamiryo, personal communication).

Five genes encoding acyl-CoA oxidases have been identified and cloned (Nicaud, personal communication).

Use of *Y. lipolytica* cells for the production of γ -decalactone from alkyl ricinoleate (a derivative of castor oil) has been patented by BASF (DE4126997, 1993), [44,38]. Other possible applica-

tion include production of wax esters from fatty alcohols [45].

The *POT1* gene encoding a catabolic 3-oxoacyl-CoA-thiolase, which catalyzes the thiolytic cleavage of 3-oxoacyl-CoA thioesters during β -oxidation of fatty acids, was cloned and characterized by Berninger et al. [46]. Disruption of *POT1* inhibited the utilization of oleate but not the elongation of externally added tridecanoic acid to higher-chain-length homologues.

3.1.3. Assimilation of alcohols

Y. lipolytica does not produce ethanol, but uses ethanol as carbon source at concentrations up to 3%. Higher concentrations of ethanol are toxic. Several NAD⁺- and NADP⁺-dependent alcohol dehydrogenases were observed in *Y. lipolytica* [32]. There probably exist two NAD⁺-dependent alcohol dehydrogenases which differ in substrate specificity. Synthesis of both enzymes seems not to be repressible by glucose or inducible by ethanol [32]. Three NADP⁺-dependent alcohol dehydrogenases were detected which exhibited different substrate specificities. The occurrence of these enzymes varies depending on growth phase and carbon source of the media [32].

3.1.4. Assimilation of acetate

Most strains of *Y. lipolytica* grow very efficiently on acetate as sole carbon source. Concentrations up to 0.4% sodium acetate are well tolerated, higher concentrations reduce the growth rate and concentrations above 1.0% inhibit the growth.

Several mutants have been isolated and characterized, which are blocked in the utilization of acetate [33,36,47–50]. Nothing is known about the uptake of acetate by this yeast. Mutants blocked in the activity of acetyl-coenzyme A synthetase were characterized by Kujau et al. [36]. Acetyl-coenzyme A is needed for the induction of the glyoxylate cycle, which is not induced in acetyl coenzyme A deficient mutants [36,47].

The induction of the glyoxylate pathway is necessary for the utilization of alkanes, fatty acids, alcohols and acetate. Isocitrate lyase, the key enzyme of this cycle, and its encoding gene is well studied [49–51,36,47,48]. The structural gene has been cloned and sequenced [52].

Dominant mutations in the gene *GPR1* (glyoxylate

pathway regulator) [36] make the cells sensitive to low concentrations of acetate or ethanol even in the presence of glucose.

3.2. Secretion of metabolites

3.2.1. Organic acids

3.2.1.1. Citrate, isocitrate. Wild type strains of *Y. lipolytica* secrete a mixture of citric and isocitric acid when grown on *n*-paraffins as carbon source: a total yield of 130% compared with the substrate and a ratio of 60:40 (citric to isocitric) was reported for strain ATCC 20114 by Akiyama et al. [53]. When the cells were supplied with monofluoroacetate, this compound was transformed into monofluorocitrate which competitively inhibited aconitase: this in turn improved the ratio of citric to isocitric acid to 85:15. In an effort to obtain a strain with low aconitase activity, a mutant unable to use citrate as carbon source was first isolated, and further mutagenized in order to obtain a fluoroacetate sensitive strain: this last strain yielded citric and isocitric acids in a ratio of 97:3, with a yield of 145% (w/w).

Tréton and Heslot [54] observed that on glucose or glycerol medium, the ratio of citrate to isocitrate was 92:8 vs 67:33 on *n*-paraffin. However the intracellular ratio of the two acids was roughly the same (90:10) on all media. These authors further showed that both acids were equally well secreted into the growth medium, but that only citric acid was re-consumed on paraffins, whereas neither was reused on glucose.

Use of mutants unable to use acetate or citrate as carbon sources has been evaluated by Wojatowicz et al. [55]. A direct selection of citrate overproducing strains has been described by McKay et al. [56].

Processes have been described for producing citric acid from glucose or *n*-paraffins in liquid cultures (for a review see Matthey, [57]) or with immobilized cells [58] but also from date coats [59], tapioca starch hydrolyzates [60] and glucose hydrol [55].

3.2.1.2. α -ketoglutarate and other organic acids.

Y. lipolytica strains grown on *n*-alkanes in the presence of a limited thiamine supply (a vitamin not synthesized by this yeast but required for α -ketoglutarate dehydrogenase activity) secrete large amounts of α -ketoglutarate. Use of a diploid strain for α -ketoglutarate production has been patented (Maldona-

do and Gaillardin, 1972). Furthermore, a haploid strain selected as a suppressor mutant of a mutation in methionine biosynthesis secretes very large amounts of this organic acid during growth on glucose (patented by Weißbrodt et al., 1988).

Production of 2-hydroxyglutaric and 2-ketoglutaric acids from glucose has been described by Oogaki et al. [61]. A process for very high yield production of isopropylmalic acid using a leucine auxotrophic strain has been patented [62].

3.2.2. Lysine

Mutations affecting all 11 biosynthetic steps of lysine except one have been identified. A single locus, *LYS1* encoding homocitrate synthase controls the first step [63,64]. The existence of isoenzymes hitherto prevented characterization of such mutants in *S. cerevisiae*. A detailed analysis of the *LYS1* gene identified homocitrate synthase as a major check point of the lysine biosynthetic pathway [65]. The *LYS1* gene has recently been cloned and sequenced [66]. Mutations at the *LYS11* locus, unlinked to *LYS1*, were initially isolated as suppressors of *LYC1* mutations [65]. They abolish completely homocitrate synthase activity. It is unknown presently if *LYS11* defines a regulator of *LYS1*, or a second subunit of homocitrate synthase.

No mutant affecting homoaconitate hydratase has been identified so far. This activity could not be purified away from aconitate hydratase. *LYS6* and *LYS7* mutations are defective in the third step of the pathway (homoaconitase), thermosensitive mutants at the *LYS6* locus identified it as homoaconitase structural gene. Homoisocitrate dehydrogenase was purified to homogeneity. Two tightly linked loci, *LYS9* and *LYS10*, actually define the same structural gene encoding a bifunctional protein involved in successive dehydrogenation and decarboxylation of homoisocitrate [67]. *LYS8* mutants are unable to convert α -ketoadipic acid into α -amino adipic acid suggesting that a single enzyme catalyzes this step in *Y. lipolytica* vs. probably two isoenzymes in *S. cerevisiae*. *LYS2* and *LYS3* are two closely linked loci, which control adenylation of α -amino adipic acid, a rate-limiting step in vivo and the only one in the pathway which is insensitive to the general control of amino acid biosynthesis [65]. The two last steps of the pathway are controlled by *LYS4*

and *LYS5*, respectively. The latter gene, encoding saccharopine dehydrogenase, has been cloned and sequenced [68,69].

Y. lipolytica uses lysine both as a nitrogen and as a carbon source, via the *N*-6-acetyllysine-5 aminovalerate pathway, like *Hansenula saturnus* [70,71]. The *LYC1* gene encoding the first step of the pathway, *N*-6-lysine acetyl transferase (LAT), was cloned and sequenced [72] and shown to be induced by lysine.

Combining mutations desensitizing homocitrate synthase (*LYS1.5*), as well as mutations simultaneously preventing lysine catabolism and relieving the lysine effect on saccharopine dehydrogenase (*lyc1.5*), yielded strains accumulating 40 times more lysine than the wild type strain [73]. No lysine secretion in the growth medium was observed, indicating that very efficient systems existed for uptake and/or retention within the cell.

Both a high and a low affinity transport system have been described for lysine uptake [74] and mutants affecting either or both systems were identified. Intracellular lysine is stored in the vacuole [75]. Mutants affecting vacuolar storage of lysine were identified among strains selected for reduced content of polyphosphates in the vacuole [76,77], although no direct relationship could be established between lysine and polyphosphate pools. A direct selection of mutations preventing lysine retention within the cell was carried out, starting with a *LYS1.5*, *lyc1.5* strain: mutants displayed a complex phenotype including reversion of the *lyc1.5* mutation, mating-type switch, and mating-type context dependent phenotypes [78].

3.2.3. Secretion of proteins

3.2.3.1. Extracellular alkaline protease (AEP).

Y. lipolytica strains grown on rich (YPD type) medium at pH 6.8 secrete large amounts (1–2 g l⁻¹) of an alkaline extracellular protease (AEP) [79,80].

AEP is encoded by the *XPR2* gene, one of at least 11 genes controlling AEP synthesis, secretion and/or activity [81,82]. The gene has been cloned and sequenced from three different strains [83–85]. AEP is a 32 kDa protease of the subtilisin family, which is intracellularly processed from a 55 kDa glycosylated precursor [84,86]. Processing includes cleavage of a 15-amino acid presequence and glycosylation of a

unique site on the propeptide (both occurring co-translationally during translocation into the endoplasmic reticulum), processive hydrolysis of a tail of 9 *N*-terminal dipeptides (X-Ala or X-Pro) by a Golgi dipeptidyl aminopeptidase, followed by the cleavage of the propeptide at a Lys-Arg junction ahead of the mature part. The endopeptidase is encoded by the *XPR6* gene, a *KEX2* homolog which has recently been cloned and sequenced [87]. The propeptide has been shown to be required for folding, transit and activation of the mature part [88,89], a finding which has since been extended to various proenzymes in *S. cerevisiae*.

Regulation of AEP synthesis is complex and reflects the carbon, nitrogen, sulfur and pH status of the cell, among others. A study of the promoter has been initiated; internal deletions and in vivo footprints demonstrate that there are two main UASs located 700 bp (UAS1) and 40 bp (UAS2) upstream from the TATA box [90]. Each UAS seems to contain targets for general transcription factors (RAP1- and possibly ABF1-like). These UAS are able to activate a minimal promoter consisting of *LEU2* TATA box and initiation site (Madzak et al., to be published). Unlinked mutations affecting transcriptional control of *XPR2* have been selected and identify at least four unlinked loci (Lambert et al., submitted). One of these identifies a zinc-finger transcriptional activator (YIRim1p) which synthesis and activity are controlled by ambient pH (Lambert et al., submitted).

3.2.3.2. Acid extracellular protease. On rich YPD medium at pH 4.0, an acid protease activity was detected in the growth medium. Three protein species were initially described, of 28, 32 and 36 kDa [91]. More recent data suggest that there is only one acid protease, which may undergo partial proteolysis in some strains and/or under certain growth conditions (Young, unpublished). Interestingly, induction of the acid protease occurs under conditions very similar to that used for inducing AEP, except for the pH of the medium. The gene encoding acid protease has been cloned and sequenced [169], and its disruption abolishes acid protease activity. It encodes a preproenzyme with an unusually large 5' upstream region. Interestingly, there seem to exist some conservation of the promoter elements of both acid and alkaline protease. Comparing the determinants of

the regulation of these two genes might thus be quite revealing.

3.2.3.3. Extracellular RNase. A secreted RNase activity is detected in *Y. lipolytica* cultures grown under conditions leading to alkaline protease secretion, the major 45 kDa protein being partially degraded by AEP into 43 kDa and 34 kDa proteins [92]. RNase is probably synthesized as a 73 kDa glycosylated precursor [92], but definite confirmation of the maturation process awaits determination of the sequence of the gene.

3.2.3.4. Extracellular phosphatases. A cell wall-bound acid phosphatase activity is induced if *Y. lipolytica* is grown on media depleted of inorganic phosphate sources. A glycosylated protein of 90–200 kDa has been purified, which is converted into a 60 kDa species upon endoglycosidase H treatment. Antibodies directed against it cross-react with *S. cerevisiae* major acid phosphatase [93]. A kinetic study of this enzyme has been published [94].

Tréton et al. [95] tried to clone its structural gene by functional complementation of a *pho5,pho3* mutant of *S. cerevisiae*. This approach failed to identify the gene searched for, but yielded *PHO2*, the gene of a secreted minor phosphatase. The *PHO2* gene product has no homology to other acid phosphatases, it corresponds to an acid phosphatase activity with a narrow substrate spectrum, the synthesis of which is induced in cells starved for inorganic phosphate.

3.2.3.5. Extracellular lipase and esterase. *Y. lipolytica* strains display a lipase activity which acts preferentially on oleyl residues at positions 1 and 3 of the glyceride. Lipase activities were examined by different investigators on different strains. It is unknown whether the reported discrepancies reflect differences in the methodologies used, or the existence of different types of strains. Peters and Nelson [96,97] described a single glucose repressible activity with a pH optimum around pH 6.2–6.5. Zviagintzeva et al. [98] also reported on a single cell wall-anchored lipase, whereas Kalle et al. [99] described two cell-bound activities: a constitutive, glucose insensitive lipase, and a second activity induced by sorbitan monooleate. Ota et al. [100] described both an extracellular activity in cultures supplemented with a protein-like fraction derived from soybean, and cell-bound lipases. The extracellular lipase required oleic acid as a stabilizer/activator, whereas the cell-

bound lipases did not, and differed in several properties from the extracellular enzyme. Sugiura et al. [101] and Ota et al. [102] solubilized two cell-bound, monomeric lipases of 39 and 44 kDa, which differed in their pH optima. Gomi et al. [103] showed that the lipase(s) exist in the cell wall as an activator-bound complex, which is rapidly dissociated upon enzyme purification. The extracellular, activator dependent activity may thus reflect release of (up to 50% of) the cell-bound activity, perhaps under conditions leading to extracellular protease(s) induction. Further details on *Y. lipolytica* lipase(s) may be found in a recent review [104].

Mutants unable to use tributyrin as a carbon source were isolated after nystatin enrichment [105], and were shown to define three complementation groups [106]. A gene encoding a secretory protein highly homologous to fungal lipases has been recently cloned in an independent approach (A. Dominguez, personal communication): this should rapidly permit assessing if there is more than one lipase activity in *Y. lipolytica*.

Y. lipolytica lipase may be of interest for synthesis of 2,4-dimethylglutaric acid monoesters, transesterification of meso-cyclopentane diols [107] or for use in the leather industry or in cheese manufacturing (German patent DD-272867).

An extracellular thermostable esterase of low molecular weight (10 kDa), specific for the 1-position of triglycerides has recently been described [108].

4. Cell biology

4.1. Peroxisome biosynthesis

Y. lipolytica grows well on oleic acid and this is accompanied by an extensive proliferation of peroxisomes. Antibodies directed against the targeting signal Ser-Lys-Leu COOH (anti-SKL) of peroxisomal proteins of several yeasts, react strongly with three peroxisomal proteins of *Y. lipolytica* [109]. Using these two features, studies on peroxisome biosynthesis have recently begun with the identification of 17 oleic acid non-utilizer mutants. Three of them were shown to affect peroxisomal assembly (*pay* mutants) by a rapid immunofluorescence assay, using anti-SKL antibodies [110]. The punctate labelling pattern

observed with anti-SKL in wild type cells was lost in the mutants, no peroxisomal structures could be observed, and two peroxisomal marker enzymes (β -hydroxyacyl-CoA dehydrogenase and catalase) failed to localize to the particulate fraction in mutant cells. Genes complementing *pay* mutations (*PAY2*, *PAY4*, *PAY5*, *PAY32*) were isolated by complementation using a genomic library made in a replicating vector. The *PAY2*, *PAY4*, *PAY5* and *PAY32* genes have been sequenced [110–113]. Pay2p is a 42 kDa peroxisomal integral membrane protein. It is essential for matrix protein import and enlargement of peroxisomes [111]. *PAY4* encodes a 112 kDa hydrophilic protein, presumably localized in the cytoplasm, which is expressed at low levels on glucose containing medium and is strongly induced upon shifting to oleic acid. Pay4p exhibits an ATP binding site and appears closely related to Pas5p of *Pichia pastoris* (59% identity) and less to Pas1p of *S. cerevisiae*, both involved in peroxisome assembly in their respective hosts [110]. *PAY5* encodes a peroxisomal integrale membrane protein homologous to the mammalian peroxisome assembly factor PAF1 [113]. *PAY32* encodes an intraperoxisomal protein of the matrix protein translocation machinery [112].

4.2. Secretion and genes involved in of the secretory pathway

An essential gene called *RYL1* has been isolated by hybridization [114], and may represent a homologue of *SEC4*, which encodes a small G protein involved in the control of secretory vesicle fusion with the plasma membrane. A homologue of *S. cerevisiae SEC14* was identified both by PCR and by functional complementation of a *S. cerevisiae sec14* mutant using a cDNA expression library of *Y. lipolytica* mRNA made in a *S. cerevisiae* expression vector [115]: unexpectedly, the deletion of this gene in *Y. lipolytica* proved to be viable and did not affect protein secretion, although recent electron microscopy data suggest that (derivatives of) the Golgi apparatus look abnormal (Rambourg et al., unpublished). This deletion however prevented the yeast-mycelium transition characteristic of this species, suggesting the existence of some link between Golgi function(s) and cellular differentiation.

Several genes controlling early steps of the pathway have been characterized. A homologue of *SEC61* (one of the subunits of the translocation pore into the endoplasmic reticulum) was cloned by PCR and shown to be highly conserved between *S. cerevisiae* and *Y. lipolytica* (68% identity at amino acid level). A homologue of *SEC62* was isolated using the cDNA expression library to complement a *sec62* thermosensitive mutant of *S. cerevisiae*. Its predicted product shows only 37% identity with its *S. cerevisiae* counterpart (D. Swennen, unpublished).

Rather close homologues of a signal recognition particle (SRP), involved in the targeting of secretory polypeptides towards the endoplasmic reticulum, have been observed in *Y. lipolytica* and in *S. pombe* [14]. On the contrary, *S. cerevisiae* SRP seems to have diverged considerably and was identified later [116]. *Y. lipolytica* SRP contains a typical 7S RNA, which can be immunoprecipitated from whole cell extracts by human anti SRP antibodies [117]. This RNA is encoded by two unlinked genes, *SCR1* and *SCR2*, whose products share 94% identity [118]. Either gene can be deleted, but the double deletion is lethal [119], indicating that essential secretory proteins strictly require the SRP dependent pathway for secretion. This also seems to be the case for AEP, whose synthesis and secretion are severely depressed under non-permissive conditions in conditional mutants of *SCR1* or *SCR2* [120,117]. Such mutants were created by site directed mutagenesis of conserved nucleotides in a loop binding Srp19p, one of the six polypeptides associated to mammalian 7S RNA. The genes encoding two of these polypeptides, Srp19 (*SEC65*) and Srp54 have been cloned and sequenced recently (M. Sanchez et al., unpublished; D. Ogrydziak, unpublished).

In an attempt to identify new genes whose product may (directly or not) interact with the SRP, mutations displaying lethality with conditional mutations within *SCR2* were selected [121]. One of these mutations identifies a gene (*SLS1*) whose product is predicted to be a resident protein in the lumen of the endoplasmic reticulum. Interestingly, deletion of this gene leads to a thermosensitive phenotype, which is further pronounced in the presence of a 7S RNA thermosensitive mutation, and results in a dramatic decrease of the amount of AEP precursor synthesis: this suggests that Sls1p may facilitate SRP-depend-

ent translocation of secretory precursors from the luminal side of the ER membrane.

4.3. Dimorphism

Wild type strains of *Y. lipolytica* exhibit various colony shapes, ranging from smooth and glistening to heavily convoluted and mat. The colony morphology is determined both by growth conditions (aeration, carbon and nitrogen sources, pH etc...) and by the genetic background of the strain. Wild type isolates may give rise to various colonial types, each giving rise to the other types: the reason for this instability, which is not seen in laboratory strains, is unknown.

Y. lipolytica is a natural dimorphic fungus, which forms yeast cells, pseudohyphae and septate hyphae [122,8]. True mycelium consists of septate hyphae 3 to 5 µm in width and up to several mm in length. Apical cells often exceed 100 µm, whereas segments are 50 to 70 µm long. There is a single nucleus per segments. Septa show a minute, ascomycete-type central pore, unusual for other filamentous yeasts, with endoplasmic reticulum extending through it from one segment to the next [123].

The proportion of the different cell forms depends on the strain used, probably accounting for the morphological differences observed at colony level. Certain conditions are known, however, which cause preferential formation of yeast cells, or induce mycelial development [124–126]. Mycelial development was inhibited by a deficiency of magnesium sulfate and ferric chloride or by the addition of cysteine or reduced glutathione. Rodriguez and Domiguez [125] could induce a complete and reproducible yeast-mycelium transition in minimal medium containing *N*-acetylglucosamine as sole carbon source, but this seems to be strain dependent, too. Guevara-Olvera et al. [126] enhanced the yeast-mycelial transition by heat shocking the cells during the inoculation in medium containing *N*-acetylglucosamine.

Some studies were done to detect physiological changes occurring during yeast-hyphae transition [127,128,126]. Comparison of the composition of yeast and hyphal cells have shown that hyphal walls exhibit a higher content of aminosugars and a reduced content of protein (Vega and Domiguez, 1986 [127]). Furthermore, ornithine decarboxylase

activity and polyamine cell pools increased in hyphal cells grown on *N*-acetylglucosamine-containing medium [126].

Mutations in the genes *SEC14* [115], *GPR1* (G. Barth, unpublished), and deletion of *XPR6* (Ogrydziak, personal communication) have strong effects on the yeast to hyphae transition. However, it is not known whether the proteins encoded by these genes are directly involved in the regulation of this transition. Morphological mutants completely unable to form hyphae, can be easily selected after visual inspection of the colonies [129].

5. Cell cycle

5.1. Mating and mating type alleles

Extensive overviews on the life cycle, including mating processes and sporulation, are given by Weber and Barth [130], Weber et al. [3], and Heslot [4].

All natural isolates of *Y. lipolytica* so far tested are heterothallic. The mating type is determined by the two alleles *MATA* and *MATB* [131]. The *MATA* allele has been cloned [132]. Similar to heterothallic strains of *S. cerevisiae* haploid and diploid cells are vegetatively stable. Kurischko and Weber [133] reported spontaneous haploidization of some diploid cells after formation of zygotes. Furthermore, it was observed in several laboratories that diploid strains spontaneously sporulate during prolonged storage on complete media. Therefore, it is recommended to prepare fresh diploid strains for genetic analysis.

Mating frequencies of natural isolates are always very low (1% viable zygotes/cell or less). Attempts to increase the mating response by inbreeding [134–136,49] failed to raise mating frequency above 15% viable zygotes/cell. Mating frequency is dependent on several parameters besides the genetic background of strains: medium, cell density, growth phase, and temperature [134,137].

5.2. Sporulation

Y. lipolytica does not require nitrogen limitation for induction of sporulation in contrast to *S. cerevisiae* and some other yeasts. Diploid strains sporulate on solid or in liquid complete medium when glucose

is exhausted. Highest sporulation frequencies were obtained with many strains if 1.5% sodium citrate was used as a carbon source [49] at temperatures of 20°C to 30°C. High sporulation frequencies may also be obtained in liquid or on solid YM and V8 media [134].

Some natural isolates of *Y. lipolytica* sporulate quite well after mating but only inbred strains form a high proportion of complete tetrads and have a satisfactory level of germinating spores.

Initial studies with Wickerham's strains were plagued by low mating frequencies, poor sporulation ability and low ascospore viability: these defects could be partially alleviated by inbreeding programmes, but no perfect set of strains could be obtained. The proportion of complete tetrads is high and the spore germination of these strains plateaued at 80%, which however makes tetrad analysis possible (summary [5]). A very efficient method for selection of spores for random spore analysis using nystatin has been developed [137].

Since genetic data are mainly gathered currently on strains of the E129–E150 series, it might be advisable to localize new genes on this map. In any case, the existence of efficient methodologies for gene transfer/replacement and for retrieval of chromosomal mutations once the gene has been cloned, makes it likely that exchange of markers between different inbred lines will be much easier than in the past.

6. Genetics and molecular biology

A fair amount of data both on genetics and molecular biology of this species had been accumulated since the mid eighties when a transformation system became available [138,139].

6.1. Mutagenesis and mutants

Several commonly used chemical and physical mutagens have been tested on wild type and inbred strains of *Y. lipolytica*, and many different mutants have been isolated and characterized. This is not the place to describe all these mutants, but some main classes and general aspects will be discussed. Results of extensive studies on cell inactivation, frequency of

mutants or of certain classes of mutants, spontaneous mutability and frequency of reversions in different strains of *Y. lipolytica* have been published [131,140,134,141,135,142].

Many mutants have been described which show nutritional requirements for most of the amino acids, adenine, uracil, and the vitamins biotin, nicotinic acid, pantothenic acid, riboflavin, and thiamine [131,134,143,135,142,4]). There are several colour mutants described which turn the colonies green, brown or red ([143,144,142]) but no red adenine requiring mutants have been isolated. Several mutants have been described which are resistant to antifungal agents or different analogues of amino acids and carbon sources [142,145,63,64]. Copper resistance varies widely among natural isolates of *Y. lipolytica*. Two tightly linked metallothionein genes, transcribed by a bidirectional promoter, have been cloned and sequenced (Dominguez et al., unpublished).

Most of the isolated mutants reverted at the expected frequency of 10^{-7} to 10^{-8} or less. However, some of the mutants reverted at much higher frequencies [142,78,36]. The reason for this high reversion has not been clarified in most cases. The cloning and sequencing of one of such a highly revertible allele (*GPR1-112*) resulted in the detection of a retrotransposon which is involved in the reversion process [146].

The nystatin method for enrichment of mutants [147,148] has been successfully adapted to concentrate auxotrophic and citrate non-utilizing mutants of *Y. lipolytica* [134,142]. Enrichment of mutants using inositol-death has been reported (DeZeeuw, personal communication).

6.2. Genome

A G+C content of 49.6–51.7% has been reported for *Y. lipolytica* [149–151]. Determination of deoxyadenosine content of exponentially growing haploid cells yielded an estimated genome size of 4×10^9 Da or about 11 Mb [134], which would be smaller than that of *S. cerevisiae*. It was later reported that different haploid strains of *Y. lipolytica* exhibit wide variations in their DNA content estimated by this method [152], but no absolute values were given. Chromosome separation by pulse field electrophoresis yielded much larger genome estimates in the

range of 12.7 to 22.1 Mb for wild type or laboratory strains [15]. A pronounced chromosome length polymorphism was observed between different isolates, as well as a variation of the number of chromosomal bands detected (4 to 6), which may reflect both gel artifacts (chromosome comigration) and aneuploidy (evidence for a duplication of the *URA3* marker on separate chromosomes was observed in a strain displaying 6 bands). Natural isolates of *Y. lipolytica* appeared to have widely divergent genetic structures, which may account for the low spore viability observed in the initial inbreeding programs.

6.3. Genetic linkage groups and chromosome maps

Based on tetrad dissection and random spore analysis, a genetic map encompassing more than 60 markers was established for a certain inbred line [135,153]. Linkage was observed 8 to 10 times more frequently between random markers than in *S. cerevisiae*, suggesting that the average recombination per kb was lower than in *S. cerevisiae*. Five linkage groups were defined at that time but linkage studies have proceeded at slow pace since that time [154]. Linkage groups were also defined for German lines [155] and for industrial strains (De Zeeuw, personal communication), but since marker exchange between the different lines is difficult, no pooled set of data is available so far. No centromere linked marker has been conclusively identified, but strains carrying centromeres tagged with a marker have been recently constructed (Fournier et al., 1993 [156]) and should help in this regard.

Inbred strains of *Y. lipolytica* tend to display 5 chromosomes in the range of 2 to 5 Mb (P. Fournier, S. Casaregola, unpublished). It should be noted that at this stage 5 is still an estimate for the chromosome number of the species: comigration may occur in this range of size. Some bands are fuzzy, which may be due to the presence of rDNA repeats, so that specific chromosome breakage will be required in order to get definite results. As expected from the study of the initial isolates from which the inbred lines were derived, each line shows a completely different chromosome pattern. An average size of 15 to 18 Mb has been estimated for inbred lines (Fournier and Nguyen, unpublished). A highly inbred strain (E150) giving consistently 5 well resolved chromosomal bands

has been selected to assign 36 cloned genes by hybridization. Ribosomal RNA genes were found on four of the five chromosomes. The linkage groups appear conserved among different independent lines.

6.4. *ARS* and centromeres

In *Y. lipolytica* an origin of replication alone is unable to maintain a plasmid extrachromosomally, but it absolutely requires a centromeric sequence [129,156].

Three different *ARS* have been isolated up to now [129,157], each carrying a centromere (*CEN*) and a nearby chromosomal origin of replication (*ORI*): *ORI3018/CEN3*, *ORI1068/CEN1*, *ORI4002/CEN4*¹. *ORI1068* and *ORI3018* have been shown by two dimensional gel electrophoresis to be included in regions of initiation of replication in the chromosome (Fournier and Vernis, unpublished), whereas all three *CEN* sequences are able to induce chromosome breakage when integrated at the *LEU2* locus. *CEN1* and *CEN3* were shown additionally to be closely linked genetically to a chromosomal centromere, and to correspond to a strong pausing site of the polymerase. *ORI* and *CEN* functions are carried by two independent regions of the original *ARS* inserts, and can be exchanged between different *ARS*: a given *CEN* sequence can thus apparently confer *ARS* function to any chromosomal *ORI*, thus opening the way to the cloning of many *ORI*. Several new *ORIs* were recently isolated from a DNA library made in a *CEN* non-transforming vector (Chasles and Fournier, to be published). One of them (*ORIX009*) was shown to be repeated at several places in the genome, another one originated from rDNA spacer.

ORI and *CEN* sequences of *Y. lipolytica* show no homology to *S. cerevisiae* or *K. lactis* corresponding sequences (nor to *S. pombe*), and the existence of a clear functional consensus for these sequences in *Y. lipolytica* has still to be assessed.

¹ Nomenclature of *ORI* and *CEN*. The *CEN* number refers to the chromosome number in strain E150 map. Four digits are used to define *ORIs*: the first refers to the chromosome number in strain E150 map, the three following are specific of each *ORI*. For *ORIs* which are repeated in the genome, the first digit is replaced by an X.

6.5. Repetitive elements

6.5.1. Ribosomal RNA and other RNA genes

Genetic data and hybridization of separated chromosomes showed that most if not all wild type isolates of *Y. lipolytica* contained several clusters of rDNA units located on 1–4 [12,158]. Up to five types of repeated units were identified in a single strain, differing in length and structure of the non-transcribed spacer DNA. All isolates so far tested showed this polymorphic rDNA structure, but one strain (ATCC 18944) was reported to contain a single type of the unit [159].

Genetic recombination within the clusters occurs during meiosis, leading to expansion or reduction of individual clusters. Thus, crossing individual strains within a given *Y. lipolytica* inbred line results in the progeny in a strain-specific reshuffling of rDNA genes supplied by each parent [158]. Strain typing can thus be done by simple inspection of hyperdense bands on digests of total chromosomal DNA. The total number of repeated units varies around a mean value of 50–60 [159]. The contribution of the rDNA repeats to interchromosome exchange seems to be low in inbred lines and in wild type isolates.

The 5S rRNA genes are not part of the rDNA clusters as in most other Saccharomycetoideae, but are dispersed throughout the genome as in *S. pombe* [12,159] and higher eukaryotic cells.

Small nuclear RNAs U1, U2, U4, U5 and U6 were analyzed by Roiha et al. [13]. Their sizes appeared much closer to that of human snRNA than to those of *S. cerevisiae*.

6.5.2. Transposons

A retrotransposon, called Ylt1, was detected in the genome of *Y. lipolytica* [146]. It is 9.4 kb long and can transpose in the genome. This retrotransposon is bounded by a long terminal repeat (LTR), the zeta element, which is 714 bp long, highly conserved, and can exist also as a solo element. Ylt1 and solo zeta elements are flanked by a 4-bp directly repeated genomic sequence. The copy numbers of Ylt1 and solo zeta are dependent on the strain examined, but at least 35 copies of Ylt1 and more than 30 copies of the solo zeta element per haploid genome have been observed.

Ylt1 belongs to the Ty3/gypsy group of retrotransposons and contains two overlapping large open reading frames (*YltA* and *YltB*). The predicted *YltA* gene product shows high similarity to retroviral Gag encoded proteins. *YltB* encodes protein domains with some similarity to retroviral gene products encoded by the *pol* gene. In the *YltB* gene, these predicted products occur in the order protease, reverse transcriptase, RNase H and integrase (Barth et al., in preparation).

6.6. Features of protein encoding genes

Several motifs similar to consensus sequences for transcriptional factor binding in *S. cerevisiae* can be observed in *Y. lipolytica* promoters, including TATA boxes, CT rich blocks, TUF/RPG or ABF1 binding sites, or UAS_{GCN} and UAS_{LEU}. The function of these sequences has not been assessed in most cases, with one exception so far [90], and the corresponding transcription factors have not been isolated. One gene encoding a transcription factor has recently become available: *CRF1*. Transcription starting points have been mapped in a few cases and often occur within a CCAA type of structure, 20–30 bp downstream from the TATA box.

Positions –1 and –3 are strongly conserved upstream of the initiator ATG: an A is observed in 12/15 cases at position –1 and in 11/15 cases at position –3. An A is found at both positions in strongly expressed genes like *XPR2*, *PGK1*, *TPH1*, *PYK1*, *ICL1* and *LEU2*.

Introns have been detected in several *Y. lipolytica* genes (5 intron containing genes among 37 known sequences). Two genes show two introns (*CDC42* and *SEC14*) separated by a short exon. The 5' end of the intron (donor site) is GTGAGTPu in all cases. The 3' internal consensus (branch site) is TACTAAC in all cases but one (cgCTAAC in the first intron of *SEC14*), and is separated by one or two nucleotides only from the 3' end of the intron: CAG.

Most *Y. lipolytica* genes show a typical signal for transcription termination TAG...TA(T)GT...TTT, which is located upstream of the site of poly A addition [115].

Codon usage appears to be different from that of *S. cerevisiae* [160] and similar to that of *Aspergillus* (A. Dominguez, unpublished).

6.7. Plasmids and VLPs

No DNA plasmid was detected in a systematic survey of 24 wild type isolates (Tréton, unpublished), but a linear dsRNA of 4.9 kb was observed in several strains [161,162]. This RNA is encapsidated within virus-like particles of 50 nm diameter. The capsid seems to be composed of two major polypeptides of 83 and 77 kDa [162,163]. Based on hybridization data, there seems to exist at least two types of dsRNAs in different strains, which show little homology to one another [162]. An additional linear dsRNA molecule of about 6 kb was detected in some strains besides the smaller 4.9 kb long dsRNA (Barth, unpublished) similar to the situation in killer strains of *S. cerevisiae*. No homology was found compared with genomic DNA of *Y. lipolytica*, nor with dsRNAs from *S. cerevisiae*. No killer phenotype associated with these VLPs could be evidenced. Curving after UV treatment did not lead to any phenotypic change.

6.8. Mitochondrial genome

The mitochondrial genome of *Y. lipolytica* has a buoyant density of 1.687 g cm^{-3} and a GC content of 24.9% [151]. It consists of a circular molecule of 14.5 mm and its restriction map was established by Wesolowski et al. [164] on strain W29. The arrangement of genes for ATPase subunits, rRNA and 4SRNA are conserved with respect to *S. cerevisiae* and *K. lactis*. Several mitochondrial genes, including those for ATPase subunit 6, 8 and 9, cytochrome oxidase subunit 3, NADH oxidoreductase (ubiquinone) subunit 4 and tRNA genes, have been cloned [165,166]. The restriction map of mitDNA seems to be well conserved among different isolates, as judged from the mobility of the corresponding hyperdense bands observed on digests of total genomic DNA stained with ethidium bromide.

A single mitochondrial mutation leading to oligomycin resistance was described by Matsuoka et al. [167]. It has been used in protoplast fusion experiments.

7. Genetic tools

Nowadays many genetic tools are available which make this yeast to an excellent model organism for addressing several fundamental questions. Powerful methods have been developed and well characterized strains and plasmids are available now for transformation, expression and secretion of foreign genes. A detailed description of these methods as well as a summary of the available strains, plasmids, cloned genes etc. is given in the review by Barth and Gaillardin [5].

8. Conclusion

The 'non-conventional' yeast *Y. lipolytica* turns out to be a superb model organism, both exquisitely original and generally meaningful. With the recent development of powerful gene amplification systems [168], the identification of a retrotransposon [146], and the characterization of strongly regulated or constitutive promoters [52,90] the stage is now set for concentrating on specific biological problems in this yeast. Investigation of mechanisms of gene expression and protein secretion for expression of heterologous genes are mainly done for biotechnological application. Fundamental studies are directed now on the structure and functioning of the genome which is in some aspects very different from what is seen in other yeasts: structure and maintenance of polymorphic and dispersed rRNA genes, absolute requirement for a centromeric function for the maintenance of extrachromosomal plasmids, and genetic structure of natural populations which consist of widely divergent haploid lines. Other aspects of *Y. lipolytica* biology have begun to be explored, like determinants of the mating type, control of the dimorphic transition from yeast to hyphae, alkane and fatty acid metabolism, structure and function of retrotransposons, glyoxylic pathway, peroxisome biogenesis, etc.

9. Note added in proof

Addition to Section 6.3. It has been shown that chromosome band no. 3 was a doublet. Therefore,

the chromosome number is six and the genome size is 18–20 Mb instead of 15–18 Mb.

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Safety Considerations with Omega-3 Fatty Acid Therapy

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It has been suggested that the potential antithrombotic effect of fish oils may theoretically increase the risk for bleeding, which may be a safety concern for individual patients. However, clinical trial evidence has not supported increased bleeding with omega-3 fatty acid intake, even when combined with other agents that might also increase bleeding (such as aspirin and warfarin). Another potential safety concern is the susceptibility of omega-3 fatty acid preparations to undergo oxidation, which contributes to patient intolerance and potential toxicity. Finally, large amounts of fish consumption may result in adverse experiences due to the potential presence of environmental toxins such as mercury, polychlorinated biphenyls, dioxins, and other contaminants. The risks of exposure to environmental toxins and hypervitaminosis with fish consumption are substantially reduced through purification processes used to develop selected concentrated fish oil supplements and prescription preparations. Thus, in choosing which fish oil therapies to recommend, clinicians should be aware of available information to best assess their relative safety, which includes the US Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) advisory statement regarding fish consumption, the meaning of certain labeling (such as "verification" through the US Pharmacopeia) and the differences in FDA regulatory requirements between nonprescription fish oil supplements and prescription fish oil preparations, and how all of this is important to the optimal treatment of patients. © 2007 Elsevier Inc. All rights reserved. (Am J Cardiol 2007;99[suppl]:35C-43C)

Does Therapy with Fish Oils Rich in Omega-3 Fatty Acids Increase the Risk for Bleeding, and Are They Contraindicated in Patients Treated with Antiplatelet and Anticoagulant Therapies?

Response: No.

Confidence/level of evidence: 2C (Table 1).

Rationale: Because of the cardiovascular benefits of omega-3 fatty acids, the American Heart Association (AHA) has recommended omega-3 fatty acid intake in the form of routine fatty fish (as well as foods rich in α -linolenic acid) for patients without atherosclerotic coronary artery disease (CAD), fish or fish oil supplements for patients with CAD, and high-dose fish oil capsules for patients with hypertriglyceridemia.¹ However, as with all pharmaceuticals, potential adverse experiences exist.

Fish oils rich in omega-3 fatty acids inhibit thrombosis, which has sometimes been suggested to account partially for the associated reduced risk for sudden cardiac death and reduced all-cause mortality.²⁻⁵ In vitro, fish oils competitively inhibit cyclooxygenase (and thus decrease the synthesis of thromboxane A_2 from arachidonic acid in plate-

lets), which leads to decreased platelet aggregation.⁶ Blood rheologic features and flow are also improved.⁷ Platelet-derived, growth factor-like protein is decreased,⁸ and synthesis of the platelet activation factor is decreased as well,^{9,10} all potentially contributing to a decrease in clinical atherothrombosis.^{11,12} Data regarding the effects of fish oils on fibrinogen and clotting factors are more limited.¹³

In contrast to this in vitro data, the in vivo human data are less definitive, and even the most common antithrombotic effects attributable to fish oils, such as a decrease in platelet aggregation, have not yet been definitively demonstrated in clinical trials.¹³ In fact, some human studies have demonstrated that fish oil omega-3 administration has no effect on platelet aggregation.¹⁴ Furthermore, low-dose fish oil therapy (<1 g/day of omega-3 fatty acids) appears to have little effect on atherothrombotic factors such as platelet-derived growth factor.¹⁵ Given that omega-3 fatty acid therapy has been shown to have favorable effects on cardiovascular end points with doses as low as 1 g/day,³ this suggests that the cardiovascular clinical benefits of fish oils are more likely related to antidysrhythmic effects compared with antithrombotic effects.^{16,17}

Nonetheless, because an antithrombotic effect is possible, it has been suggested that fish oils could potentially increase the risk for bleeding.¹⁸ Furthermore, although the consumption of fish high in omega-3 fatty acids may decrease the risk for thrombotic stroke, it has been suggested that fish oil omega-3 therapy may actually result in a slightly higher risk for hemorrhagic stroke.¹³

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Table 1
Scales for assigning confidence and type of evidence* codes to the answers given to task force questions

Scale	Description
Confidence	
1	Very confident
2	Confident
3	Marginally confident
4	Not confident
Type of evidence	
A	Well-designed RCTs, including RCTs conducted in patients who have reported adverse experiences
B	- Single RCT with a highly statistically significant result - Well-conducted retrospective case-control studies with adverse experiences as primary end points - Managed care claims database analysis with a highly statistically significant result
C	- Reports to regulatory agencies judged to exceed population averages and reporting bias - Multiple case studies with nonblinded dechallenge and rechallenge - Strong trends, not reaching statistical significance, for safety issues in large RCTs - Well-conducted prospective cohort study, giving a result that is statistically well above population average - Metabolic or clinical surrogate studies
D	- Undocumented opinion of experienced research investigators and clinicians - Poorly controlled or uncontrolled studies - Nondefinitive evidence from regulatory agency reporting systems or managed care claims databases
U	Unknown, no appropriate evidence, or evidence considered subject to bias

RCT = randomized controlled clinical trial.

* Support for evidence for or against the contention that a potential human adverse experience is related to lipid-modifying medications.

So the question is as follows: Do the potential antithrombotic effects of fish oils rich in omega-3 fatty acids pose a significant risk for increased bleeding in clinical practice, particularly when combined with other antiplatelet or anticoagulant therapies? In short, the clinical trial evidence suggests that if such an increased bleeding risk exists, the risk is very small and not of clinical significance.

Clinical trials have shown high-dose fish oil omega-3 fatty acid consumption to be safe, even when concurrently administered with other agents that may increase bleeding, such as aspirin and warfarin.^{12,19,20} In fact, in certain clinical settings, it could be argued that the reported antiatherothrombotic effects of fish oils rich in omega-3 fatty acids are a benefit that outweighs the unproved bleeding risks, especially in select patients at high risk for thrombosis,^{11,21,22} which may include patients with acute atherosclerotic CAD.²³ Finally, it is important to note that on the basis of existing clinical trial outcomes data, the AHA has recommended omega-3 fatty acid consumption (about 1 g of eicosapentaenoic acid [EPA] plus docosahexaenoic acid [DHA] per day in the form of fatty fish or fish oil supplements) in patients with documented atherosclerotic CAD,¹

most of whom also would likely be treated with antithrombotic agents such as aspirin.

Recommendations to healthcare professionals (Table 2): Clinical trial data are lacking for every conceivable patient situation. From a practical standpoint, clinicians should be aware of regulatory recommendations included in the prescribing information of Omacor (Solvay Pharmaceuticals, Inc., Marietta, GA),²⁴ the only prescription fish oil preparation.²⁵ The drug interaction with anticoagulant section states, "Some studies with omega-3-acids demonstrated prolongation of bleeding time. The prolongation of bleeding time reported in these studies has not exceeded normal limits and did not produce clinically significant bleeding episodes. Clinical studies have not been done to thoroughly examine the effect of Omacor® and concomitant anticoagulants. Patients receiving treatment with both Omacor® and anticoagulants should be monitored periodically."

Thus, a commonsense approach in treating patients routinely consuming significant amounts of fish oils, including prescription fish oils or supplements (in each case >1 g/day of EPA and DHA), would be to use similar general guidelines applicable to other anticoagulants. For example, it

Table 2

Recommendations to healthcare professionals regarding the safety of fish oil therapy

- The clinical trial evidence does not support an increased bleeding risk with fish oil therapy, even when used in combination with other agents that may increase bleeding (such as aspirin and warfarin).
- It is reasonable to monitor patients treated with fish oils and anticoagulants for potential bleeding adverse experiences; however, it is unclear if more monitoring is required than what would otherwise be done with patients administered anticoagulants alone.
- Fish oils should probably be discontinued during acute bleeding episodes, such as hemorrhagic stroke.
- The decision to discontinue fish oils days before an invasive procedure at high risk for bleeding complications should be based on weighing the unproved potential increase in bleeding risk versus the potential reduction in atrial fibrillation before certain procedures, such as coronary artery bypass surgery.
- Rigorous purification processes involved in fish oil manufacturing reduce the risk of fatty acid oxidation, hypervitaminosis, and exposure to environmental toxins.
- Clinicians and patients should be aware of the variance in the purification processes among different fish oil manufacturers.
- Because fish oil supplements are generally regarded as safe, they are not subject to FDA premarket and approval requirements.
- If a product has the "USP-Verified" mark on its label, the manufacturer has met voluntary USP standards, which include initial and ongoing determinations to ensure that (1) what is on the label is in fact in the bottle (all the listed ingredients in the declared amounts), (2) the supplement does not contain harmful levels of contaminants, (3) the supplement will break down and release ingredients in the body, and (4) the supplement has been made under current good manufacturing practices.
- Some fish oil manufacturers advertise voluntary compliance with the standards set by the CRN, which suggests that the fish oil manufacturers are adhering to a voluntary monograph developed by an association of manufacturers in the dietary supplement industry that specifies quality standards for fish oil supplements marketed in the United States.
- Claims of a fish oil supplement being "pharmaceutical grade" have little meaning regarding safety and have even less meaning with regard to efficacy, unless the fish oil preparation has been approved by the FDA as a prescription pharmaceutical.
- Prescription fish oil preparations undergo the same rigorous FDA regulatory requirements as other prescription pharmaceuticals, with regard to both efficacy and safety.
- One of the most common pitfalls in the day-to-day, clinical use of fish oil therapy is the sense among patients that all fish oil therapies are the same. Clinicians need to educate patients of the wide variance in fish oil therapies regarding efficacy, tolerability, and perhaps even safety. For example, the efficacy of fish oil therapy is most dependent on the amount of omega-3 fatty acids (such as EPA and DHA) in each capsule, not the total amount of fish oil concentrate. Thus, to achieve the same level of omega-3 fatty acid intake, patients may have to take as many as 11 capsules of some fish oil supplements to match the same amount of omega-3 fatty acid intake as 4 fish oil capsules of prescription fish oil.

CRN = Council for Responsible Nutrition; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; FDA = US Food and Drug Administration; USP = United States Pharmacopeia.

would seem prudent to discontinue high-dose fish oil consumption or supplementation in the setting of an acute bleeding illness, such as during and immediately after a hemorrhagic stroke, or in patients at high risk for hemorrhagic stroke. Another practical clinical example might be the perioperative management of patients who undergo some major surgeries. It is true that the even infusion of fish oils after major abdominal surgery (as occurs through total parenteral nutrition) does not appear to result in clinically significant bleeding and has been suggested to be safe with specific regard to coagulation and platelet function.²⁶ Nonetheless, some clinicians might consider discontinuing fish oil therapy 4–7 days before planned invasive procedures with the highest risk for bleeding complications, as is often recommended with aspirin, warfarin, and clopidogrel.^{27–29} However, in the case of fish oils, the unproved benefit of stopping fish oil therapy to reduce a theoretical increase in bleeding risk should be weighed against the potential benefits of fish oil therapy in reducing atrial fibrillation, when administered ≥ 5 days before major procedures such as coronary artery bypass surgery.²⁷

Postoperatively, clinicians should consider the potential antithrombotic and cardiovascular advantages of restarting fish oil therapies, given that thrombotic and cardiovascular events are often among the most common complications following many major surgeries.

Do Prescription and/or Supplement Omega-3 Fatty Acid Products Contain Excessive Vitamins or Toxins, Such as Mercury, Polychlorinated Biphenyls, Dioxin, or Other Contaminants, in Sufficient Concentrations to Pose a Potential Health Risk?

Response: No.

Confidence/level of evidence: 2B (Table 1).

Fish oil oxidation: Clinically, the most common intolerance to fish oils is a "fishy" smell and aftertaste, as well as fishy eructations.^{25,28,29} Fish oils are naturally highly unstable and susceptible to oxidation, which accounts for their rancid conversion and contributes to patient intolerance. Fish oil oxidation may also increase the risk for toxicity.³⁰ One of the more common ways to reduce fish oil oxidation and thus reduce fish oil rancidity, maintain freshness, and increase shelf life is to add the antioxidant vitamin E to supplements. The concurrent use of vitamin E with fish oil consumption may also overcome the oxidative stresses to the body that are observed when fish oils without vitamin E are consumed.³¹ Another practical maneuver to improve tolerance and reduce the fishy aftertaste is to refrigerate fish oil supplements before administration. Alternatively, some patients may find that they prefer liquid or powdered fish oil preparations.

Perhaps the most important measures that can be taken to reduce the fishy smell and taste complaints, intolerances,

and particularly the potential toxicities of fish oil therapy involve the manner in which the fish oil products are manufactured, specifically regarding purification processes directed at the removal of toxins, non- ω -3 fatty acids, oxidized substances, and other undesirable byproducts.²⁵ In fact, it could be argued that when a patient describes a fish oil supplement as having a strong, rancid fishy smell and taste, this may suggest that the supplement was poorly purified by the manufacturer or has expired or become oxidized and is potentially toxic.

Hypervitaminosis: Hypervitaminosis is another potential toxicity that may occur with the excessive consumption of fish oils containing high concentrations of fat-soluble vitamins D and A, as might occur with excessive cod liver oil consumption. Very high vitamin D intake can cause hypercalcemia,³² but this has not been a widely described consequence of any fish oil consumption, including cod liver oil ingestion. Similarly, studies suggest that vitamin A toxicity is rare when fish oils are administered in oil-based preparations.³³ Nonetheless, the excessive intake of vitamin A (particularly in water-miscible, emulsified, and solid forms) has been described to cause yellowish discoloration of the skin (carotenemia); possibly an increased risk for lung cancer³⁴; teratogenic effects in pregnant women³⁵; severe anemia and thrombocytopenia in infants³⁶; toxicities to the musculoskeletal, neurologic, and gastrointestinal system in children³⁷; bone pain; and osteoporosis, with an increased risk for bone fractures in older subjects.³⁸

In the early 1900s, rickets (a bone disease due to vitamin D deficiency) was rampant among poor children in the United States and England who were living in industrialized cities amid polluted, smoky skies. Vitamin D deficiency ensued because sunlight is needed to metabolize vitamin D. Around this same time, cod liver oil (an ω -3 fatty acid) was found to be useful to prevent rickets because it contained substantial amounts of vitamin D.³⁹ Cod liver oil also was used as a supplement to treat a variety of many pediatric ailments, partly because it also contained high doses of vitamin A, and vitamin A deficiency was thought to contribute to blindness and immune deficiencies and thus an increased risk for infections. Thus, it was not uncommon for parents to administer cod liver oil routinely to their children and for pediatricians to recommend infant formulas that included cod liver oil.

However, since the fortification of infant milk preparations with vitamins D and A in the 1930s, the use of cod liver oil to prevent rickets is no longer common in the United States.³⁹ US pediatricians no longer routinely recommend cod liver oil for infants, although cod liver oil remains a component of some "homemade" infant formulas. Thus, clinicians should be made aware that cod liver oil supplementation is still common in some populations, such as those in northern Europe, and thus may represent at least a potential risk for hypervitaminosis.

Environmental toxins: High fish oil intake through the consumption of large amounts of fish may present a risk for increased environmental toxin exposure.⁴⁰⁻⁴² For example, excessive mercury exposure may originate from industrial sources (such as coal-fired power plants, waste incinerators, and certain factories and mining operations). Once airborne, the mercury pollutants fall to the ground in rain or snow, become deposited in water bodies, and are subsequently converted by bacteria into methylmercury, which is highly toxic to humans.⁴³ Larger and older fish may have more time to bioaccumulate mercury from their food (and through their gills) than smaller and younger fish.⁴⁴ Furthermore, large predatory fish near the top of marine food chains may accumulate more mercury than fish lower in the marine food chain through the consumption of small fish, resulting in biomagnification. Mercury poisoning through fish consumption has resulted in various neuropsychiatric signs and symptoms, including constriction of the bilateral visual fields, paresthesias of the extremities and mouth, ataxia, incoordination, tremor dysarthria and auditory impairments, severe neurologic damage to children born to mothers with toxic mercury exposure,^{43,45} and other signs and symptoms.⁴⁶ As a testament that mercury exposure poses real risks to populations, in 2004, the US Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) issued an advisory statement recommending that women who may become pregnant, women who are pregnant, breast-feeding mothers, and young children avoid eating some types of fish and eat fish and shellfish that are lower in mercury.⁴⁷ However, the totality of evidence supports that the benefits of fish intake generally exceeds the potential risks, including intake in women of childbearing age, except for a few selected fish species.⁴⁸ It is noteworthy that these recommendations apply only to fish oil intake through the consumption of fish. With regard to fish oil intake through select fish oil supplements, testing has shown that the level of mercury (and other environmental toxins) is very low or negligible.^{49,50} This is probably due to 2 factors. First, the oxidized mercury released from power plants and potentially washed into local water bodies by rainfall is water soluble.⁵¹ Thus, although mercury toxicity may sometimes be a potential issue with the consumption of the flesh of fish, oxidized mercury is insoluble in oil and thus would not be expected to represent a significant toxicity risk with the intake of the oils of fish. Second, selected fish oil supplements undergo extensive purification processes to remove environmental and other toxins, with prescription fish oil preparations undergoing even more rigorous regulatory processes to achieve FDA approval as prescription pharmaceuticals.

Before 1980, polychlorinated biphenyls (PCBs), colorless and odorless chemicals, were used widely in flame retardants, pesticides, paints, varnishes, inks, lubricants, and insulation related to electrical equipment, such as transformers. Since then, PCBs have been banned by most countries.⁴³ As with mercury, PCBs are toxic to humans as well

as to fetuses.^{43,52} PCBs are a probable human carcinogen. Acute PCB toxicity most commonly manifests as chloracne lesions or rashes. Other manifestations of PCB toxicity include elevated liver enzymes and possibly an increased risk for goiter. Finally, exposure to PCBs in utero may result in genetic alterations or mutations in a fetus's reproductive organs (feminized unborn males, intersexed fetuses, or the in utero development of male and female reproductive organs), and in utero or breast milk exposure to newborns may result in neurodevelopmental delays, impaired cognition, and immune deficiencies.⁴³

Other environmental toxins include organochlorine (OC) pesticides, which were sprayed on crops and forests and released into the air, water, and soil. One of the best known OCs is dichlorodiphenyltrichloroethane (DDT), which has been banned in the United States since the 1970s. DDT is highly toxic to fish, and its widespread use was a major reason for the near extinction of the bald eagle in the 1950s. Although the clinical manifestations of OC toxicity may not be as clear as other toxins, OCs may cause abnormalities in liver enzymes and chloracne⁵³ and contribute to neurologic impairment, such as Alzheimer disease.⁵⁴

Dioxins, which are another family of chemical compounds called polychlorodibenzodioxins (sometimes grouped as part of OCs), are a byproduct of industrial processes involving chlorine, such as waste incineration, chemical and pesticide manufacturing, and pulp and paper bleaching. Dioxin was the primary toxic component of Agent Orange, a defoliant used during the Vietnam War, and is considered a likely carcinogen. Dioxin toxicity is commonly manifested by chloracne and has also been suggested to contribute to the onset of diabetes mellitus, endometriosis with infertility, abnormalities in thyroid function, and impaired neurodevelopment of infants.⁵⁵

Just as with mercury, fish consumption has sometimes been associated with toxicities from all of these environmental toxins.^{40–43,55,56} Thus, manufacturers of selected fish oil supplements have implemented various purification processes and quality controls designed to reduce the risk for exposure to environmental toxins. Largely through these quality measures, reviews of 5 commercial fish oil supplements have concluded that some of the more common fish oil supplements contain fewer toxins than fish and that fish oil supplements may be preferable to fish consumption as a therapeutic source of omega-3 fatty acids.^{50,56}

Recently, a prescription omega-3 fatty acid fish oil preparation has been approved for the treatment of hypertriglyceridemia (Omacor). In multiple clinical trials of this prescription omega-3 fatty acid agent,²⁵ no cases of hypervitaminosis or illnesses due to exposure to environmental toxins were reported. This safety profile is likely owing to an extensive purification process resulting in no detectable concentrations of heavy metals, halogenated polycarbons, and dioxins; as well as <0.05% of trans-fatty acids.²⁵

Recommendations to healthcare professionals (Table 2): In choosing the most appropriate fish oil therapy to recommend to patients, clinicians should be aware of potential fish oil toxicities and know which fish oil manufacturers have adequate purification processes to minimize these potential toxicities. This can present a challenge, because although the FDA has regulatory mechanisms to ensure the safety of prescription products,⁵⁷ no such FDA regulatory mechanisms are in place for “dietary supplements.” Agents classified as dietary supplements do not require product registration, manufacturer registration, pre-market approval, or the mandatory reporting of adverse events and require only limited safety-related labeling. From a practical standpoint, this means that manufacturers of dietary supplements are not required to provide evidence of efficacy, safety, or manufacturing standards before marketing products.⁵⁸ Although it has been suggested that this lack of oversight and inadequate regulation may pose a risk to the health and safety of the public,⁵⁸ and although organizations such as the American Medical Association (AMA) have advocated that dietary supplements be regulated to the same standards as prescription and over-the-counter drugs,⁵⁹ in the current regulatory environment, clinicians should be aware of the quality assurance practice guidelines for fish oil products, self-established by their manufacturers, before recommending any dietary supplement.

For example, specific FDA current good manufacturing practices are required for the prescription pharmaceuticals and describe procedural protocols that help ensure acceptable quality.⁶⁰ Current good manufacturing practices are currently under development for dietary supplements (Table 3). In the interim, fish oil supplements are included on the list of substances that the FDA designates as “generally regarded as safe,” which means that according to qualified experts, fish oils have adequately been shown to be safe under the conditions of their intended use. As with other products generally regarded as safe, fish oils supplements are not subject to the premarket review and approval requirements of the FDA. Nonetheless, some fish oil manufacturers include in their advertisements that fish oil supplements have been designated by the FDA as generally regarded as safe and that their manufacturing processes are in voluntary compliance with current good manufacturing practices. With regard to efficacy (and safety) in the clinical use of fish oil supplements, the FDA has determined that the following statement is acceptable, provided that fish oil supplement manufacturers do not recommend or suggest in their labeling a daily intake that exceeds 2 g/day of EPA and DHA: “Consumption of omega-3 fatty acids may reduce the risk of coronary heart disease. The FDA evaluated the data and determined that, although there is scientific evidence supporting the claim, the evidence is not conclusive.”⁶¹

Because of the recognition that “faith” alone is not sufficiently reassuring to many clinicians or patients regarding medicinal product quality and safety, some fish oil supplement manufacturers elect to pursue “USP-Verified” marks

Table 3
A comparison of the US Food and Drug Administration's (FDA) regulatory mechanisms to help ensure the safety of products

Product Class	Product Registration	Manufacturer Registration	Premarket Approval of Products	Specific Good Manufacturing Practices	Voluntary Postmarket Adverse Event Reporting System	Mandatory Manufacturer Reporting of Adverse Event	Safety-Related Labeling Requirements
Dietary supplement				Under development	✓		Some
Conventional foods				✓	✓*		Some
Food additive			✓	✓	✓	Some	✓
Monograph drugs†	✓	✓		✓	✓		✓
New drug application drugs‡	✓	✓	✓	✓	✓	✓	✓
Infant formula	✓	✓		Proposed in 1996	✓	✓	✓

* The FDA does not collect or evaluate all adverse events on all conventional food. Excluded from this system are the investigations the FDA conducts after food-borne illness outbreaks.

† Monograph drugs are typically over-the-counter drugs that must adhere to specific safety standards set out for each ingredient and do not undergo clinical testing.

‡ A new drug application must be submitted to the FDA for all prescription drugs and some over-the-counter drugs before market. This application must include data that demonstrate the safety and efficacy of the product.

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on their labels. A USP-Verified mark indicates compliance with standards set by the United States Pharmacopeia (USP), an independent, not-for-profit organization established in 1820 that has quality standards enforceable by the FDA and sets the legally recognized standards for identity, strength, quality, purity, packaging, and labeling.⁶² Many clinicians are aware of USP monographs, mostly issued for prescription or over-the-counter products and sometimes for dietary supplements as well. A USP monograph is a descriptive document that typically contains the following information about a product: descriptive information (graphic formula, chemical formula, molecular weight, chemical name, and chemical abstracts registry number), percentage of the active ingredient in the product, a discussion of product packaging and storage, USP reference standards, any official revisions to the monograph, tests used to identify the product, melting temperature range, amount of water, residue on ignition, and a list of tests and procedures to assess toxins and impurities. No official USP monograph currently exists for nonprescription fish oil supplements.

Beyond describing monograph specifications of products, the USP is also engaged with the verification of products, such as through the voluntary Dietary Supplement Verification Program.⁶³ The presence of the distinctive USP-Verified mark on its label signifies that the USP has rigorously tested and verified a supplement to ensure that (1) what is on the label is in fact in the bottle (all the listed ingredients in the declared amounts), (2) the supplement does not contain harmful levels of contaminants, (3) the supplement will break down and release ingredients in the body, and (4) the supplement has been made under current good manufacturing practices.^{63,64} If a product has a USP-Verified mark on its label, the manufacturer is legally responsible for meeting USP standards. Two separate USP verifications exist. One distinctive USP-Verified mark is "Ingredient Verified." In this case, the USP has performed testing at the request of the manufacturer to verify the consistent quality of active and inactive ingredients. This mark is used mainly by manufacturers. The other distinctive USP-Verified mark is "Dietary Supplement Verified," indicating that testing has been done to ensure the integrity, purity, dissolution, and safe manufacturing of a dietary supplement.⁶³ This mark is for mainly for consumers. At the time of this writing, only a few fish oil supplement manufacturers have voluntarily requested and subsequently received either of these USP-Verified designations.⁶⁴

To assist in the establishment of quality and safety standards that might form the basis of an official USP monograph for the class of nonprescription fish oil supplements, the Council for Responsible Nutrition (CRN) developed a voluntary monograph aimed at the goal of "raising the bar" through quality standards for fish oil supplements marketed in the United States. The CRN is an association of ingredient suppliers and manufacturers in the dietary supplement industry founded in 1973 for the purpose of "improving the environment for member companies to responsibly market

dietary supplements by enhancing confidence among media, healthcare professionals, decision makers and consumers”⁶⁵ and has encouraged the incorporation of this voluntary monograph into existing standards established by organizations such as the USP, as well as other organizations such as the American Oil Chemists Society and the Association of Official Analytical Chemists International. Some fish oil manufacturers claim voluntary compliance with the proposed CRN fish oil monograph.

Currently, the USP is providing its verification mark on the basis of a proposed monograph, which was derived from the CRN monograph, as well as other resources. The establishment of an official USP monograph for fish oil therapy is imminent. However, even with the establishment of an official USP monograph, clinicians and patients should understand that no USP designation exists to qualify omega-3 fish oil supplements as “pharmaceutical grade,” as claimed by some fish oil manufacturers in their advertisements. The USP has no standards that defined the term “pharmaceutical grade”; thus, any labeling of an omega-3 fish oil supplement as “pharmaceutical grade” as it pertains to the USP is misleading. In fact, it might better be considered as blatantly inaccurate, unless the fish oil formulation has gone through the rigorous processes and oversight required to receive approval as a prescription pharmaceutical from an established regulatory agency such as the FDA⁵⁷ (Table 1).

So the answer as to whether prescription and/or supplement omega-3 fatty acid products may contain excessive vitamins or environmental toxins in sufficient concentrations to pose a potential health risk is dependent almost entirely on the purification process. With nonprescription fish oil supplements, because no FDA regulatory oversight exists to ensure a lack of included toxins, clinicians and patients must rely on their faith in the voluntary commitment to quality on the part of manufacturers or ensure that the fish oil supplement manufacturers have complied with accepted quality assurance standards, as denoted by labeling such as “USP-Verified.” The only caveat here is that any such labeling does not verify, or even address, the degree of efficacy of a supplement. For efficacy information, a label must be read to assess the amount of EPA and DHA within a fish oil dietary supplement, which can then be used to determine the anticipated therapeutic response. This is important because although the front of the bottles of some fish oil supplements may list the contents as having “1,000 mg of fish oil concentrate,” the labeling on the back of the bottles may reveal that the amounts of the actual omega-3 fatty acids (eg, EPA and DHA) are much less, often as low as 300 mg per fish oil capsule. In this case, from a practical standpoint, a patient would have to consume 11 fish oil capsules containing 300 mg EPA and DHA to achieve the same omega-3 fatty acid intake as 4 tablets of prescription fish oil (which contains 840 mg of EPA and DHA).²⁵

If a fish oil preparation has received FDA approval as a prescription pharmaceutical, clinicians and patients can be assured that the manufacturer has met rigorous FDA regu-

latory requirements with regard to both efficacy and safety when used in the clinical setting and at the doses in compliance with the prescribing information.

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Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects

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Abstract: Because of their characteristic living environments, marine organisms produce a variety of lipids. Fatty acids constitute the essential part of triglycerides and wax esters, which are the major components of fats and oils. Nevertheless, phospholipids and glycolipids have considerable importance and will be taken into account, especially the latter compounds that excite increasing interest regarding their promising biological activities. Thus, in addition to the major polyunsaturated fatty acids (PUFA) such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, a great number of various fatty acids occur in marine organisms, e.g. saturated, mono- and diunsaturated, branched, halogenated, hydroxylated, methoxylated, non-methylene-interrupted. Various unprecedented chemical structures of fatty acids, and lipid-containing fatty acids, have recently been discovered, especially from the most primitive animals such as sponges and gorgonians. This review of marine lipidology deals with recent advances in the field of fatty acids since the end of the 1990s. Different approaches will be followed, mainly developing biomarkers of trophic chains in marine ecosystems and of chemotaxonomic interest, reporting new structures, especially those with biological activities or biosynthetic interest. An important part of this review will be devoted to the major PUFA, their relevance to health and nutrition, their biosynthesis, their sources (usual and promising) and market.

Keywords: Lipids · Fatty acids · Marine organisms · Biomarkers · Nutrition

Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects

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Abbreviations

AA	arachidonic acid 20:4(n-6)
DHA	docosahexaenoic acid 22:6(n-3)
EPA	eicosapentaenoic acid 20:5(n-3)
FA	fatty acid
FATM	fatty-acid trophic markers
GC/MS	gas-chromatography mass spectrometry
PUFA	polyunsaturated fatty acids
LC-PUFA	long-chain polyunsaturated fatty acids
VLC-PUFA	Very-long-chain polyunsaturated fatty acids
MUFA	monounsaturated fatty acids
NMI	non-methylene-interrupted fatty acids
NMID	non-methylene-interrupted dienoic fatty acids
PL	phospholipids
SFA	saturated fatty acids
TL	total lipids
EEA	essential fatty acid
TAG	triacylglycerols
SCO	single-cell oil

1**Introduction**

Lipids are major sources of metabolic energy and essential materials for the formation of cell and tissue membranes. They are very important in the physiology and reproductive processes of marine animals and reflect the special biochemical and ecological conditions of the marine environment [1-3]. The interest of chemists, biochemists and biotechnologists in lipids and fatty acids (FA) from marine animals and algae has been stimulated, in particular, by the recognition that polyunsaturated fatty acids (PUFA) are important to human health and nutrition. They are required for reproduction and growth. The relative proportion and composition of FA in marine organisms are characteristic for every species and genus, and also depend on environmental conditions. Several comprehensive reviews are available on marine FA, their occurrence, their roles and the methods used in their analysis [4-8].

The principal role of neutral lipids, which in marine organisms consist predominantly of triacylglycerols (TAG) and wax esters, is as an energetic reserve of FA that are destined either for oxidation to provide energy (ATP) or for incorporation into phospholipids. Phospholipids are the building blocks for the membrane lipid bilayer. FA provide the hydrophobic interior of all cell membranes, forming an impermeable barrier to water and polar molecules and separating the cell contents from the extracellular medium. The physical properties of the membrane are determined by the individual lipids

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within the FA components of the lipids and their interaction with sterols and proteins. While their function as structural lipids in membranes has been known for a long time, ceramides and glycosyl ceramides (glycosphingolipids) play an important role in many fields of cell biochemistry, such as molecular recognition. In addition, ceramides from marine organisms have excited great attention as signal transducers, and some of them have been recognized as possessing antimicrobial and cytotoxic activities. Marine glycosphingolipids, chiefly isolated from sponges, show interesting biological activities such as immunomodulation and antitumoral activity [9]. The fatty acyl chains linked to these classes of compounds are often commonly occurring but several new and original structures have been reported recently [10, 11]. Arising from alpha-glycosphingolipids isolated from marine sponges, the simplest (KRN 7000) is being considered as an anticancer agent ([12], see Bourguet-Kondracki & Kornprobst, this book).

Fat not only provides energy, it facilitates the absorption of fat-soluble vitamins (vitamins A, D, E, and K), and plays an important role in the production and regulation of eicosanoids. In addition, lipid class and FA compositions can be used to understand and identify food web interactions.

Different approaches can be applied in this field of marine lipidology, focused on FA, mainly:

- searching for new FA structures
- evaluating new sources of major PUFA of biological interest
- evaluating their role in cell membranes
- investigating biosynthetic pathways
- developing trophic and/or chemotaxonomic biomarkers in ecosystems.

The rapid development of excellent analytical methods, especially gas liquid chromatography coupled to mass spectrometry (GC/MS), which can deal with complex mixtures, has also been a major contributing factor to the progress in the chemistry and biochemistry of marine lipids [5, 6, 13].

As the principal producers in the marine environment, microalgae support both pelagic and benthic food webs, and their lipids and FA are been extensively studied. Microalgae are known to have different FA compositions depending on their taxonomic position [14, 15]. At the next trophic level, zooplankton form an essential link between primary producers and higher-order consumers. Very recently, a complete review was devoted to fatty-acid trophic markers (FATM) in the pelagic marine environment [8]. The FATM concept is based on the observation that marine primary producers lay down certain FA patterns that may be conservatively transferred through aquatic food webs. Thus, they can be recognized in their primary consumers. The next step is concerned with the dynamics of FA in fish, which catabolize and transform dietary FA.

Marine invertebrates, especially sponges, have proved to be a rich source of many unusual FA. Sponges are very ancient animals with special structural features, particularly the cell membrane, which have allowed their adaptation

to often precarious environments. These primitive organisms are difficult to classify due to the few available useful morphological characteristics. The use of taxonomic knowledge allows investigation to be focused on genera that offer the great potential in metabolites of biological interest. Recently, a comprehensive taxonomy was published, which provides the state of the art [16]. Representatives of the Coelenterate phylum have remarkable peculiarities in their FA composition. Some species contain unusual FA or unusual concentrations of common components.

The main purposes of this paper are to illustrate the molecular biodiversity in marine lipids (from the first planktonic marine producers to fish) and, in another part, to focus on the most important PUFA (sources, biosynthesis, economy). This work has been done by considering the most interesting advances in marine lipidology since the end of the 20th century. Thus, without covering the subject exhaustively, this review deals with FA from total lipids or those specifically linked to a particular lipid class such as TAG, wax esters, ceramides, polar lipids and some additional atypical secondary metabolites. Thus, unusual or novel FA, and FA that are interesting due to their applications in chemotaxonomy, biosynthesis and as biomarkers, will mainly be taken into account.

2

Nomenclature of fatty acids

According to the international nomenclature, the position of the first double bond is given by the (n-x) notation, counting the number of carbon atoms from the methyl end. For instance, 18:4(n-3) identifies an FA with 18 carbon atoms and four double bonds, the first double bond occurring after the third carbon atom [4, 7] (Fig. 1). An additional PUFA is depicted in Fig. 1 (middle), namely 24:6(n-3).

According to an alternative notation, the locations of the double bonds are counted from C-1 (the carboxyl group). Thus, 18:4(n-3) is designated 6,9,12,15-18:4. This latter notation will be used in particular in the case of non-methylene-interrupted fatty acids (NMI FA), such as 7,13,17-20:3 shown above, the most commonly encountered among these being the dienoic acids (NMID) (Fig. 1, bottom).

The configuration of double bonds, generally assumed as *cis* (Z) in natural compounds, must be indicated in other cases. The positions of a methyl branch or another group is indicated by the number of the carbon atom on which the chain is substituted (e.g. 10-methylhexadecanoic acid or simply 10-Me-16:0; 2-hydroxydocosanoic acid or 2-OH-22:0; 6-bromo-5,9-heptacosadienoic acid or 6-bromo-5,9-27:2).

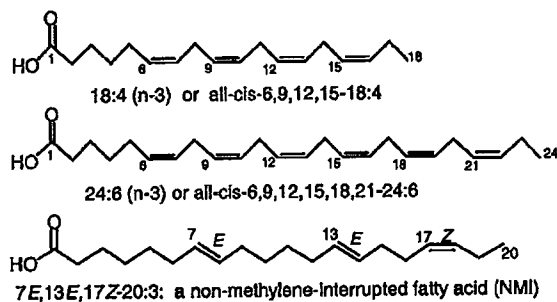


Fig. 1 Examples of numbering and designation of fatty acids (FA)

3

Fatty-acid biosynthetic pathways in primary producers

The basic processes of FA biosynthesis are summarized in Fig. 2. The *de novo* biosynthesis of FA follows the common pathway with major end products being mainly 16:0 and also 14:0, 18:0 and 20:0 (also produced by chain elongation) [8].

Then, an aerobic desaturation is catalyzed by the enzyme Δ^9 -desaturase to give rise to 16:1(n-7), 18:1(n-9) and 20:1(n-11). Generally, only plants are capable of biosynthesizing *de novo* (n-3) and (n-6) PUFA (Fig. 2A). Oleic acid 18:1(n-9) is the precursor of all (n-3) and (n-6) PUFA. The next double bonds are introduced to form 18:2(n-6) and 18:3(n-3). Through appropriate desaturations and chain elongations, 18:2(n-6) may be further converted to 20:4(n-6) (AA), and 18:3(n-3) to EPA. DHA is obtained via C_{24} PUFA intermediates rather than direct elongation of EPA, according to the so-called Sprecher pathway [21–23]. This biosynthetic scheme is typically observed in dinoflagellates, in which FA such as 18:4(n-3) and DHA are often dominant. Furthermore, the biosynthetic pathway producing 16:4(n-1) from 16:0 is characteristic of diatoms [8, 24]. The *de novo* biosynthesis of long-chain monounsaturated FA (MUFA) typically pronounced in calanoid copepods is showed in Fig. 2B. Biosynthetic considerations will be developed in detail in the last part of this review.

4

Marine bacteria and cyanobacteria

Marine bacteria are known for their role in nutrient cycling and the degradation of organic matter [8]. The roles of bacteria in marine food webs has two aspects, firstly as primary food sources, and secondly as components of

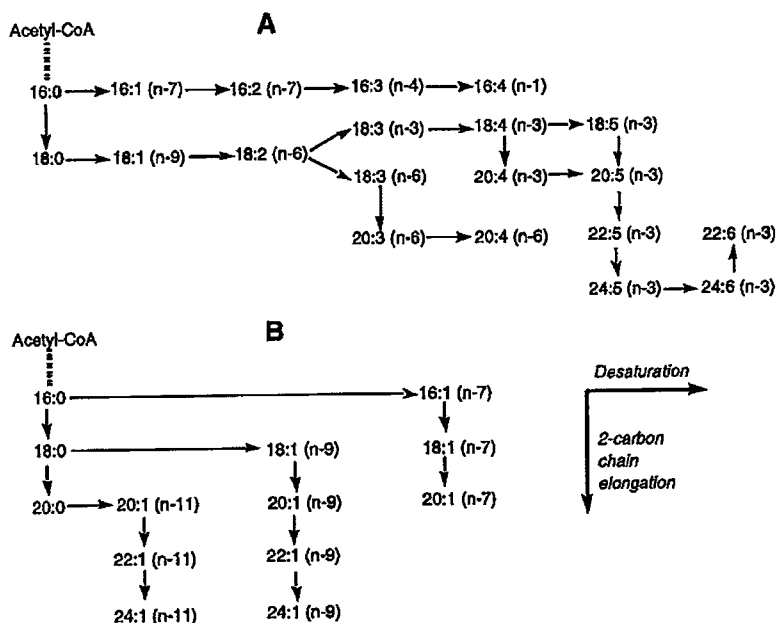


Fig. 2 Major pathways of FA biosynthesis in marine algae (a), modified after Gurr & Harwood [17] and Cook [18], and herbivorous calanoid copepods (b), modified after Sargent & Henderson [19] and Kattner & Hagen [20]. Extracted from Dalsgaard et al. [8]

the commensal microbial communities of marine animals [25]. Marine heterotrophic bacteria are abundant in sediments and as colonizers of settling particulate matter following plankton blooms [26]. That observation explains why the FA composition of marine bacteria has mainly been studied by geochemists [26–29]. Bacteria incorporate FA mainly in PL. Bacterial FA are commonly saturated (SFA) and monounsaturated (MUFA), ranging from C_{10} to C_{20} , whereas PUFA are quite rare. Bacterial FA biomarkers are typically odd-numbered, branched *trans*-unsaturated and cyclopropyl FA such as 15:0, 17:0, *iso*- and *anteiso*-branched SFA and MUFA, 10-methyl-16:0 ([8] and references therein).

Furthermore, deep-sea bacteria and several bacterial strains (*Pseudomonas*, *Vibrio*) have been shown to be capable of producing (n-3) PUFA, as recently reviewed [24, 25, 30–32]. Very little is known about the biosynthesis of PUFA in bacteria. EPA and DHA in bacteria are contained within phospholipids rather than TAG. Therefore, marine bacteria seem to be of limited use as a source of oils rich in (n-3) PUFA [24]. (see the chapter entitled “Promising sources of marine LC-PUFA”)

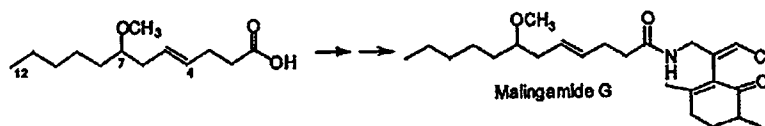


Fig. 3 Malingamide G and its potential precursor 7-methoxy-dodec-4-enoic acid

Cyanobacteria, a class of photosynthetic prokaryotes occurring in the phytoplankton, produce C_{18} PUFA esterified to polar lipids, but they do not biosynthesize EPA or DHA [24]. EPA production was obtained by a transgenic marine cyanobacterium carrying a plasmid containing the essential open reading frames for EPA synthesis [33].

Fatty-acid amides are widespread in nature [34]. They are incorporated into some lipid classes such as ceramides, glycosphingolipids and various *N*-acylated lipid molecules. A new diacylgalactolipid was isolated from the marine cyanobacterium *Oscillatoria* sp. comprised of 9,12-octadecadienoyl and 4-hexadecenoyl chains [35]. Cyanobacteria of the genus *Lyngbya* are a rich source of bioactive secondary metabolites including fatty-acid amides [36]. A series of biologically active malyngamides has been identified from marine cyanobacteria with a mid-chain methoxylated fatty acyl chain [37–40]. As an example, Malyngamide G and the new 7-methoxydodec-4(*E*)-enoic acid (its possible precursor) were isolated from *Lyngbya majuscula* collected off the French Mediterranean coast (Fig. 3).

Both these compounds are non-cytotoxic to KB cells and show immunosuppressive activity [39]. In several other malyngamides, the fatty-acid amid is 7-methoxy-tetradecen-4-enoic.

5

Phytoplankton

Primary producers provide the basic FA patterns in marine food webs. They consist of macroalgae and phytoplankton, which mainly comprise microalgae and photoautotrophic bacteria. Phytoplankton in the pelagic environment comprises mainly Bacillariophyceae (diatoms), Dinophyceae (dinoflagellates) and Prymnesiophyceae. Algal FA are biosynthesized in the chloroplasts comprising the thylakoid membranes, and are chiefly esterified to glycolipids rich in (n-3) PUFA. During the exponential growth phase of phytoplankton blooms, carbon fixed through photosynthesis is allocated to growth and cell division rather than lipid storage. Therefore, the level of glycolipids is particularly high in this phase, and the proportion of (n-3) PUFA can reach 50% of total lipids [8, 14, 41, 42]. It is well known that plants are usually the only organisms that can biosynthesize de novo the acids 18:2(n-6) and 18:3(n-3).

These FA and their principal derivatives (e.g. AA, EPA and DHA) are essential constituents of heterotrophic organisms. Thus, algae occupy a central position within marine food webs.

As shown in a recent review, FA patterns can be used as potential taxonomic markers regarding the presence and combinations of certain FA that can be characteristic of particular algal classes, whereas individual FA cannot be used as indicators of particular algal species [43]. This approach has been developed in the case of diatoms and dinoflagellates, two important classes in marine environments. Thus, high values of $16:1(n-7)/16:0$ (typically > 1), and $\sum C_{16}/\sum C_{18}$ have been associated with a dominance of bacillariophytes [28, 29, 44]. Furthermore, high values of $18:5(n-3)/18:3(n-3)$ and $\sum(C_{18} \text{ PUFA}, C_{22} \text{ PUFA})$ have been associated with dinophytes [45]. Bacillariophytes can be distinguished from dinophytes by means of high values of $\sum C_{16}/\sum C_{18}$ together with low values of $18:5(n-3)/18:3(n-3)$ [45]. This criteria can be reinforced by the examination of the ratio $22:6(n-3)/20:5(n-3)$ [28]. A value ≥ 1 indicates a predominance of dinophytes, while a value < 1 indicates a predominance of bacillariophytes. The lipids of diatoms, characterized by high levels in EPA and the absence of DHA, are also rich in C_{16} PUFA [24].

If investigations of FA isolation and purification have principally been carried out on $(n-3)$ PUFA, there are other potentially interesting FA commercially unavailable. Thus, the acids $16:3(n-4)$, $16:2(n-4)$ and $16:2(n-7)$ were isolated as methyl esters by means of liquid chromatography using a porous graphitic-carbon phase [46].

The taxonomy of the Raphidophyceae is still uncertain and needs the help of chemotaxonomic data, such as FA composition, in order to distinguish correctly the genera [45–49]. The FA compositions of twelve raphidophyte strains were established and were very similar to previous data [47]. The major PUFA were EPA (14.8–24.5%) and $18:4(n-3)$ (12.0–26.6%, with an exception at 0.3%) [49]. High levels of free FA were observed in lipids of *Fibrocapsa* species (23.6–37.9%). FA profiles allowed clear discrimination between the genera. By using a selected EPA-deficient mutant of *Porphyridium cruentum*, it has been demonstrated that TAG of the red microalga *P. cruentum* can contribute to the biosynthesis of eukaryotic galactolipids [50]. FA biomarkers were used to investigate the biogeochemistry of a former blue-mussel aquaculture site and the high levels of PUFA found indicated a substantial phytoplankton source (diatoms and dinoflagellates) [29]. FA composition of toxic microalgae have been determined to detect useful biomarkers in screening seawater seafood samples [51]. Two *Pseudo-nitzschia* species were studied and both displayed similar FA compositions typical of diatoms, including $16:1(n-7)$, $16:2(n-4)$ and EPA as major unsaturated FA. $16:4(n-1)$ occurred in both species and therefore could be used as a signature compound in differentiating toxic *Pseudo-nitzschia* from other diatoms [51]. Furthermore, the possibility of using boiling water to deactivate lipolytic enzymes, as previ-

ously found [52], was confirmed, and it was suggested that some mechanisms of PUFA degradation was also inhibited [51].

The FA compositions of microalgae have been shown to change in response to changes in salinity. Green unicellular microalgae of the genus *Dunaliella* are known for their capability to grow at high salinities up to salt-saturated water [53]. The major unsaturated FA in two *Dunaliella* isolates originating from Antarctic hypersaline lakes were 16:4(n-3), 18:3(n-3), 18:2(n-6), 18:1(n-9) and 16:1(n-7). The results suggest that the appropriate environment necessary for the growth of these halophilic species is a certain level of osmotic pressure in the medium [53].

High levels of DHA are noticeable in the lipids of the Dinophyceae, another major component of marine phytoplankton, together with significant amounts of EPA, 18:5(n-3) and 18:4(n-3) [24, 54]. Thus, it seems possible that dinoflagellates are capable of synthesizing DHA through the chain-shortening of 24:6(n-3), since a similar mechanism could produce 18:5(n-3) from EPA [24]. The presence of EPA and also DHA and 18:5(n-3) has been linked to potential toxicity in a raphidophyte *Heterosigma* as well as in a dinoflagellate *Gymnodinium* [55, 56]. It was suggested that the mode of action in the ichthyotoxicity of these harmful bloom-forming flagellates is correlated to oxygen radical formation [49]. Two *Gymnodinium* species and several other dinoflagellate species were found to contain unusual octacosapolyenoic FA, namely 28:7(n-6) and 28:8(n-3) at levels up to 2.2% of total FA [57] (Fig. 4).

VLC-PUFA have also been found in some autotrophic and heterotrophic lower organisms such as microalgae, fungi, sponges and bacteria [59], and most of them are either saturated or monounsaturated. Such FA were identified in Baltic herring, namely 28:7(n-3) and 28:7(n-6) [58]. Furthermore, the two *Prorocentrum* species studied were found to contain 18:4(n-3) (12.7–15.3%), 18:5(n-3) (36.4–37.6%) and DHA (18.3–22.0%) [57, 58]. 28:8(n-3) was also identified in a commercially available oil, used as dietary supplements of PUFA for humans, from the heterotrophic dinoflagellate *Cryptocodinium cohnii* [60]. Furthermore, these unusual octacosapolyenoic FA also occurred as minor components in lipids of all five dinoflagellates studied for fatty acid and sterol compositions [57]. The major unsaturated FA were as followed: 18:4(n-3) (2.5–11.5%), 18:5(n-3) (7.0–43.1%), EPA (1.8–20.9%) and

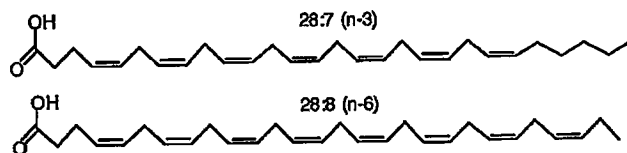


Fig. 4 Octacosapolyenoic FA from dinoflagellates

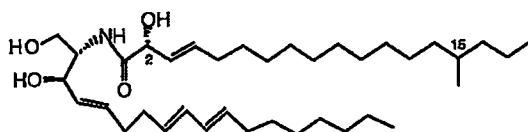


Fig. 5 New ceramide from the dinoflagellate *Coolia monolis*

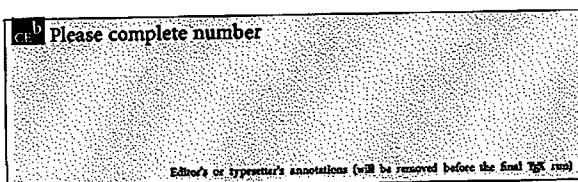
DHA (9.9–26.3%). A new ceramide isolated from the epiphytic dinoflagellate *Coolia monolis* bears a novel fatty acyl moiety, namely 2-hydroxy-15-methyl-3-octadecenoyl [61] (Fig. 5).

The fatty acid composition of polar lipids and TAG was determined in different types of symbiotic dinoflagellates isolated from several hermatypic corals from a fringing reef in Japan [15]. 18:4(n-3) (10.0–26.2%) and DHA (10.6–17.8%) were found as major PUFA in polar lipids of symbiotic dinoflagellates isolated from all species studied. In addition, polar lipids of dinoflagellates from *Millepora intricata* were different from those originating from other corals in that they contained high amounts of 18:5(n-3) (8.7%) and 22:5(n-6) (10.3%). It was suggested that FA might provide useful information on possible taxonomic differences among symbiotic dinoflagellates [15].

6

Macroalgae

FA in marine algae have attracted considerable attention among researchers because they can produce significant amounts of interesting PUFA and provide useful distinguished features of chemotaxonomic value [62]. The data available on lipids from macroalgae have been reviewed recently [8]. Three classes are mainly concerned: Chlorophyceae, Rhodophyceae and Phaeophyceae. FA from 11 macroalgae from the French Brittany coast were studied [63]. More recently, Li et al. [62] reported the FA compositions of 22 species of marine macrophytes, collected from the coast of the Bohai Sea belonging to the three aforementioned algal classes. These algae have FA patterns typical of red, brown and green algae from other regions. In general, red algae from the Bohai Sea contained high levels of C₂₀ PUFA, primarily that of EPA (up to 37.5%) and AA (up to 29.4%). The main difference in the FA compositions between red and brown algae was that the latter were richer in C₁₈ PUFA, especially in 18:4(n-3) (up to 20.1%). Seven of the ten brown algal species studied also contained EPA as a major component, accounting for 8.4–24.2%. Green algae studied had the highest level of C₁₈ PUFA, mainly 18:3(n-3) (20.5–27.2% of total lipids) and 18:4(n-3), and the lowest level of C₂₀ PUFA [62]. Interestingly, another study of FA composition of



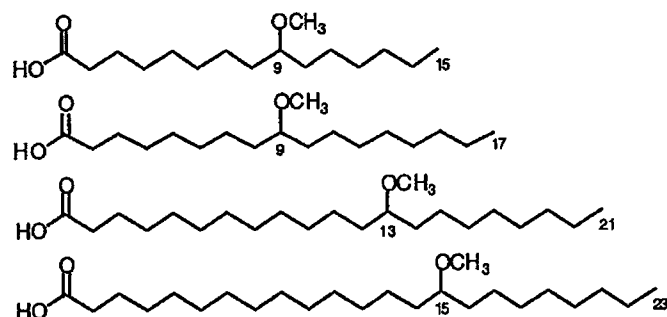


Fig. 6 New methoxylated FA from lipids of *Schyzimonia dubyi*

19 species of the same algal classes, collected on the Pacific coast of North California gave rise to very similar conclusions [64]. Red Californian algae contained AA (5.3–23.4%) and EPA (27.8–45.4%). Brown algae contained 18:4(n-3) (3.6–18.6%) and EPA (3.1–15.5%). Two of the three green algae studied contained 16:4(n-3) (13.6–16.2%) and 18:4(n-3) (12.1–22.1%). Both these studies show that red, brown and green algae have distinguished FA profiles that do not depend on the geographical location of the algae and that have a chemotaxonomic significance for seaweeds [62, 64]. A recent comparative study of FA composition of Arctic and Antarctic macroalgae considered their use as indicators of phylogenetic and trophic relationships [65]. Several eicosanoids, metabolites of AA, such as hydroxytetraenoic acids, associated with prostaglandins, were identified in a Japanese red alga *Gracilaria asiatica* [66].

Methoxy FA are not very widespread in nature [67, 68]. Several mid-chain methoxy FA have been reported only in certain microorganisms and marine cyanobacteria (genus *Lyngbya*), as seen above. Four novel mid-chain methoxy FA (16% of total acid mixture) were identified in lipids from a red alga as 9-MeO-15:0, 9-MeO-17:0, 13-MeO-21:0 and 15-MeO-23:0 acids [69], as depicted in Fig. 6.

These algal lipids contained C₁₄–C₂₈ SFA, accounting for 77%, and hydroxylated FA, but no PUFA. Furthermore, marine sponges have provided new 2-methoxy long-chain acids that will be presented in a following chapter.

7

Zooplankton

During the last decade, zooplanktonic organisms from Arctic and Antarctic waters have given rise to intense research, especially on lipids [70–90].

These investigations have highlighted the general lipid characteristics in high-latitude zooplankton communities, such as copepods and ctenophores, in terms of food webs and biomarkers. The Southern Ocean has a complex food web including planktivorous herbivores (krill, salps, copepods) that are fed upon by fish, squid, seals and whales [83,87]. Krill (*Euphausia superba*) provide 30–90% of the diet for these carnivores and have an estimated standing stock biomass of 200–400 million metric tons. This high biomass reflects krill's ability to adapt to marked seasonality in food supply. Krill are primarily herbivores feeding on phytoplankton in the summer. In the winter krill feed mainly on ice algae [87,89]. Krill lipids have been intensively studied because of their commercial interest and undisputed importance in the Southern Ocean, while the importance of gelatinous organisms, such as salps, ctenophores and medusae, is now recognized in marine pelagic ecosystems.

As the best-studied group of zooplankton with respect to the FA trophic-markers concept, herbivorous calanoid copepods have been mainly examined. They dominate the zooplankton biomass in high-latitude ecosystems and accumulate large lipid reserves. In addition, the FA characteristics of omnivorous and carnivorous copepods have been summarized, especially using FA as markers of carnivory [8]. Moreover, calanoid copepods are themselves important producers of specific FA and fatty alcohols (from wax esters). They play an important role in polar food webs and provide higher trophic levels with a lipid-rich high-energy diet [71]. However, similarities and differences between species emerge more clearly if the major lipid classes are analysed separately to determine their compositions. Phospholipids and the storage lipids (TAG and wax esters) are expected to exhibit strong compositional differences, which may provide additional information on their structural and energetic functions [71].

The compositions of wax esters, TAG and phospholipids in nine Arctic and Antarctic copepods have provided evidence of energetic adaptations with similarities and differences between the species [71]. The wax esters of the herbivorous species were clearly characterized by the long-chain monounsaturated FA 20:1(n-9) and 22:1(n-11), whereas the omnivorous and carnivorous species usually had high relative amounts of 18:1(n-9). The phospholipids contained very high levels of PUFA, especially 22:6(n-3). The phospholipid FA compositions in both Arctic and Antarctic species were found to be very similar. This extremely high degree of unsaturation (EPA and DHA together accounted for 46–60% of the total phospholipid FA) is quite unusual. In a recent investigation, the variation in the FA content was related to the spatial distribution of krill and the available diet as well as to maturity and sex [83]. *E. superba* are known as essentially herbivores when phytoplankton is abundant, but they can be omnivorous if algal biomass becomes relatively low. Three regionally groups of krills were considered. Krills from one group, almost exclusively juveniles, were surviving in the region characterized by

lowest algal biomass and had probably resorted to carnivory on PUFA-rich copepods [83]. The latter krill had unusually high level of PUFAs, mainly 18:4(n-3), EPA and DHA. Changes in lipid composition of the Antarctic *E. superba* were investigated regarding the influence of geographical location, the sexual maturity stage and the distribution among organs [73].

Lipid metabolism of *E. crystallophias* and its ecological implications have been studied because this euphausiid is the dominant krill species in high-Antarctic waters. Thus, it has to cope with the most extreme environmental conditions of all euphausiids [76]. Wax esters were the primary storage lipids and accounted for up to 55.6% of total lipids, including 18:1(n-9) and 18:1(n-7) as major components (together up to 90% of total wax esters acids). 16:0, EPA, DHA and 18:1(n-9) were the major phospholipid FA components.

Little is known about lipids and FA of a member of gelatinous zooplankton, the salps (Tunicates). The pelagic tunicate *Doliolotta gegenbauri* is abundant in the North Atlantic and occurs in frequent blooms [74]. Polar lipids were the dominant lipid classes. The FA composition was largely dominated by EPA (13–14%) and DHA (27–30%).

8

Marine Invertebrates

8.1

Sponges

Marine sponges are the most primitive multicellular animals and contain many new metabolites, including lipids, in particular glycolipids and phospholipids [91–97]. Thus, sponge lipids are one of the richest source of unusual FA. Sponges are very ancient animals with special structural features in their cell membranes, in particular phospholipid FA and sterols, since sterol-phospholipid interactions are assumed to play a major role in cell membranes [91–93]. Furthermore, sponge classification needs to be supported by chemotaxonomic criteria, in particular regarding FA. Recently, a comprehensive taxonomy was published, which provides the state of the art [16]. Marine invertebrates, e.g. sponges, are filter feeders and consequently they can be associated with microorganisms. Thus, particular FA appear as biomarkers for such organisms. It has been chosen here to focus on the most unusual recent data, such as unsaturated or branched patterns.

8.1.1

$\Delta 5,9$ fatty acids

Major sponge phospholipid FA include very low amounts of the usual methylene-interrupted PUFA, or none at all, but high levels of very-long-

chain acids (C_{23} to C_{34} , representing up to 80%); these are the so-called demospongiic acids. Sponges (Demospongia) are a source of novel FA structures, especially unusual long-chain $\Delta 5,9$ FA with no counterpart in the terrestrial world, with sometimes a third double bond or a bromine atom [93, 96–102]. In each set of sponge phospholipids studied, about 50 to 70 FA were identified. Some sponges contain up to fifteen $\Delta 5,9$ FA in their phospholipids and, in several cases, one of them accounted for more than 50% of the total phospholipid FA mixture [99, 102]. Such NMI FA are showed in Fig. 7.

Approximately twenty $\Delta 5,9$ unsaturated FA were found in these sponges [102]. It is generally admitted that $\Delta 5,9$ demospongiic acids are biosynthesized by Demospongiae through short-chain unsaturated FA, mainly from exogenous $\Delta 9-16:1$ [93]. The sponge *Geodinella robusta* was very interesting in that it contained an unusually high level of free FA, mainly with $\Delta 5,9$ unsaturation, including the rare *anteiso*-5,9-24:2 acid (19.5% of the total free FA), and the new *iso*-5,9-24:2 acid (30%) [103]. Mixtures of these latter compounds showed cytotoxic activity against mouse *Ehrlich carcinoma* cells and a hemolytic effect on mouse erythrocytes [103]. The FA of total lipids from *Halichondria panicea* included $\Delta 5,9,19-26$ a major component (33%), and six other $\Delta 5,9$ FA [104]. Several common PUFA, such as AA, EPA and DHA, occurred in this sponge. The 5,9,23-triacontatrienoic methyl ester, isolated as a natural compound by bioassay-guided fractionation from the marine sponge *Chondrilla nucula*, is an elastase inhibitor with the potential to be a therapeutic agent in some diseases such as pulmonary emphysema and chronic bronchitis [105]. The 5,9,21-30:3 sponge acid was reported as a DNA topoisomerase inhibitor [106].

Brominated FA from marine invertebrates have also been reviewed [100]. Recently, a comprehensive review of natural halogenated FA included those from marine algae and invertebrates [36]. *Cinachyrella* sponges from the Red Sea also contained three 6-brominated acids with the new 6-bromo-5,9-nonacosadienoic [102]. 6-Bromo-5,9,24-27:3 and 6-bromo-5,9,24-28:3 acids have been isolated from the sponge *Xestospongia* sp. [107, 108]. It should be noted that the latter FA, and the 6-bromo-5,9-heptacosadienoic acid, displayed moderate activity against murine leukaemia L1210 cells and against human carcinoma KB cells [108].

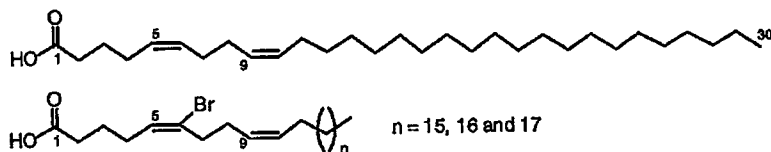


Fig. 7 $\Delta 5,9$ fatty acids from *Cinachyrella* sponges

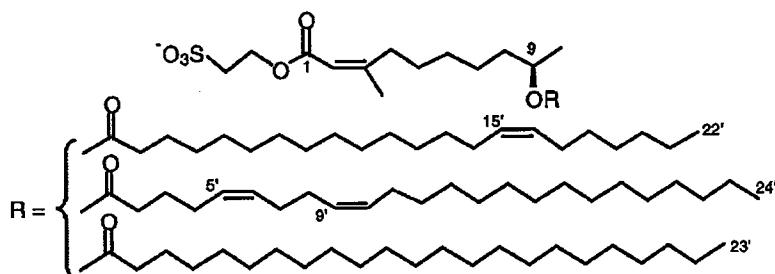


Fig. 8 Irciniasulfonic acid from *Ircinia* sp.

Nevertheless, it was demonstrated that sponges are not the only source of these unusual $\Delta 5,9$ FA since they have been observed in zoanthids and sea anemones [109, 110], gorgonians [100, 111]. Thus, the novel *iso*-5,9-pentadecadienoic acid was isolated from *Eunicea succinea* and prepared by synthesis [111]. In addition, new 6-bromo-5,9-eicosadienoic acid was identified from an anemone and a zoanthid [110].

A novel FA analogue, named irciniasulfonic acid, has been isolated from the Japanese marine sponge, *Ircinia* sp. Its structure consists of three different kinds of acids, i.e. common FA, a novel unsaturated branched C_{10} FA and an isethionic acid. Irciniasulfonic acid and deacyl irciniasulfonic acid reverse multidrug resistance in human carcinoma cells caused by overexpression of membrane glycoprotein [112] (Fig. 8).

8.1.2

Branched fatty acids

Branched FA including isoprenoid acids have often been found in marine sponges. The main isoprenoid FA are 4,8,12-trimethyltridecanoic and phytanic acids [95, 96, 101, 102]. As a recent example, phospholipid FA of two sponges of the genus *Cinachyrella* from the Red Sea were studied and compared with previous results for other *Cinachyrella* sponges [102]. Five SFA not hitherto found in nature were identified, namely 17-methyl-24:0, 18-methyl-24:0, 18-methyl-25:0, 18-methyl-26:0 and 18,24-dimethyl-26:0 acids [102] (Fig. 9).

The rare 10,13-dimethyl-14:0 and the new 9,13-dimethyl-14:0 were identified in marine sponges [102, 114]. The occurrence of bacteria in sponges is supported by the huge levels of phosphatidylglycerol and phosphatidylinositol that are characteristic of bacterial membranes [93]. In addition, several branched short-chain FA were identified, which are typically of bacterial origin, such as *iso*- and *anteiso*-15:0, *iso*- and *anteiso*-17:0, 10-methyl-16:0, 13-methyl-16:0, 10-methyl-18:0, and 11-methyl-18:0 acids. The new branched

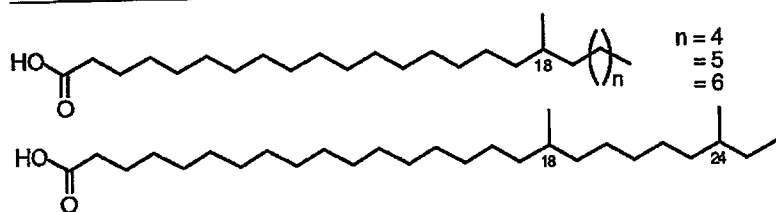


Fig. 9 New long-chain branched FA from *Cinachyrella* sp.

long-chain acids probably arise from shorter precursors of exogenic origin through a homologation process [102]. Other sponges were found to be rich in such branched FA, including additional branched FA, 3-methyl branched short-chain acids and the new 8,10-dimethyl-16:0 acid [101, 115–117]. The lipids of the sponge *Hymeniacidon sanguinea* from the Black Sea contained 73 FA, including the novel 13-methyl-20:0, 15-methyl-22:0 and 3,13-dimethyl-14:0 [116]. The compositions of lipids in this sponge collected from two locations with different ecological conditions (Canary Islands and Black Sea) were compared [94, 116]. The significant differences in the structures and relative concentrations of the typically bacterial FA, and the occurrence of cyclopropane-containing FA only in the Canary Islands sponge, suggests that the symbiotic bacteria in the two species are different [116].

A most interesting finding in *Callyspongia fallax* was a series of *iso* monoenoic branched-chain C_{15} – C_{17} and double bonds at either $\Delta 4$, $\Delta 5$, $\Delta 6$, $\Delta 7$, or $\Delta 9$ [117]. The lipids of the stromatoporoid *Astrosclera willeyana* (a “living fossil” sponge) and the demosponge *Agelas oroides* contained complex isomeric mixtures, at large amounts, of branched FA including *iso-lanteiso*-branched FA and abundant mid-chain branched acids present in the C_{15} to C_{25} range. These compounds are most likely derived from specific heterotrophic bacterial symbionts [118].

8.1.3

Methoxylated fatty acids

Methoxylated FA are relatively rare in nature and limited to primitive organisms such as cyanobacteria, bacteria and sponges [66, 101, 117, 122]. The first naturally occurring α -methoxylated FA were found in phospholipids from the sponge *Higginsia tethyoides*, which contained saturated, monounsaturated and diunsaturated α -methoxylated FA with chain lengths between 19 and 28 carbon atoms [121, 122]. Methoxylated lipids have been recently reviewed with an emphasis on the alkylglycerol ethers and FA bearing the methoxy group in the alkyl chain [119]. In that recent review, 29 α -methoxylated acids were listed [67]. These phospholipid α -methoxylated FA share the common molecular properties of possessing the *R* configuration

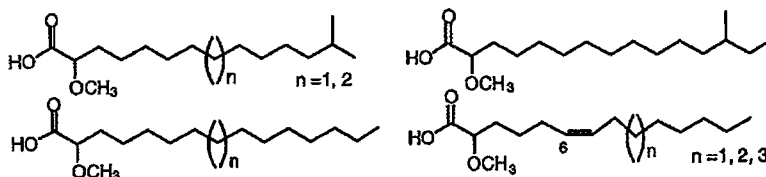


Fig. 10 Some α -methoxylated FA identified in Caribbean sponges

at the chiral center [123]. While the first very-long-chain α -methoxylated FA (C_{19} – C_{28}) could have arisen from sponge cells, recent examples of short-chain analogs (C_{14} – C_{18}) are postulated to originate from bacteria in symbiosis with sponges. A novel series of α -methoxylated FA have been reported from Caribbean sponges from the genera *Amphimedon*, *Callyspongia* and *Spherospongia* (Fig. 10) [122, 123]. Various syntheses of methoxy FA have been performed [124].

8.1.4

Acetylenic fatty acids

Acetylenic FA have been found in sponges [125, 126]. Brominated C_{16} , C_{18} , and C_{20} acetylenic FA have been reported from sponges of the genera *Xestospongia* and *Oceanapia* [127]. An already known brominated acetylenic acid was isolated from the sponge *Xestospongia testudinaria* by bioassay fractionation (A_1 adenosine receptor affinity), as shown in Fig. 11 [128].

This FA was the active compound. Two novel steryl esters with bromo-acetylenic chains that were also isolated were found to be inactive. A C_{14} acetylenic acid from the sponge *Oceania* sp. showed significant antimicrobial activity against various bacteria and fungi [129]. Recently, new acetylenic FA from the *Stelletta* sponge species exhibited weak cytotoxicity against a human leukemia cell line [130] (Fig. 12). The second compound from *Stellata* is a symmetric dimer of the first, and is the first example of a sponge metabolite possessing an acid anhydride functionality.

In a recent work, a sulfated C_{24} acetylenic FA, namely callysponginol sulfate A, was isolated by bioassay-guided fractionation from the Japanese sponge *Callyspongia truncata* (Fig. 13) [131].

Callysponginol sulfate A is the first example of an acetylenic acid containing a sulfate group from marine organisms. These compounds inhib-

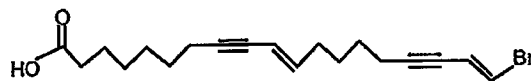
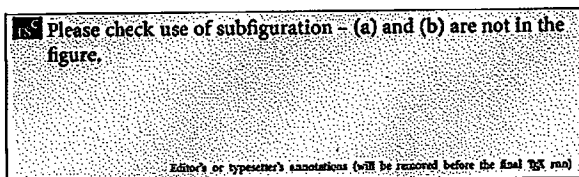


Fig. 11 Biologically active FA from *Xestospongia testudinaria*



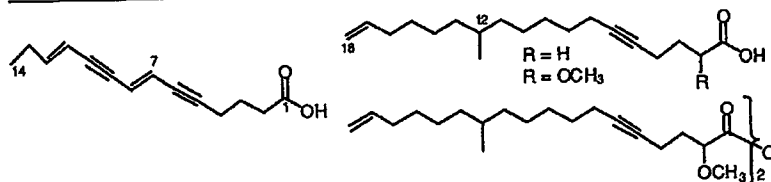


Fig. 12 New acetylenic FA from (a) *Oceanapia* and (b) *Stellata*

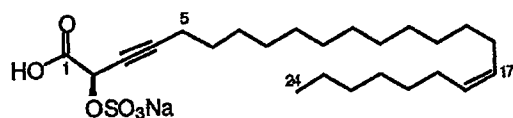


Fig. 13 Callysponginol sulfate A from *Callyspongia truncata*

ited membrane type 1 matrix metalloproteinase (MTP1-MMP), one of the key enzymes involved in tumor growth, migration, angiogenesis, invasion and metastasis [131]. Recently, new lysophosphatidylcholines and monoglycerides were reported from *Stelletta*, which include acetylenic fatty acyl chains and dimethyl branched chains [132].

Ceramides have received increasing interest because of their various properties, including antifungal and antimicrobial activities. Recently, several new ceramides have been isolated from various marine sponges including *Haliclona tenuiramosa* (Fig. 14A) [133, 134]. They contain the usual saturated C_{20} to C_{26} fatty acyl chains. Biofouling organisms such as barnacles, mussels and macroalgae cause serious damage to ship's hulls and aquaculture nets. Thus, it is very important to identify nontoxic alternatives to the organotin compounds currently used. The sponge *H. koremella* provides a new ceramide with activity as an antifouling substance against macroalgae (Fig. 14B) [133]. This work showed that the length of the acyl residue seems to be important for the antifouling activity of ceramides. Recently, a mixture of ceramides has been isolated from the red alga *Ceratodictyon spongiosum* containing the symbiotic sponge *Sigmatocia symbiotica* [135]. Their non-hydroxylated acyl chains ranged from C_{22} to C_{24} . In addition, a unique 24-methylenecholesteryl ester (19:0) was characterized in these organisms.

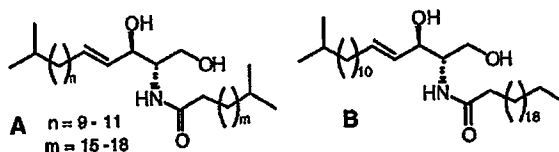


Fig. 14 Ceramides from the sponges *Haliclona tenuiramosa* (a) and *H. koremella* (b)

Glycosphingolipids (GSL) are ubiquitous membrane constituents in animals that play fundamental roles in major phenomena such as cell-cell recognition and antigenic specificity. GSL from marine sponges possess interesting immunostimulatory and antitumor activities [9-11, 136-141]. Sponges are a very rich source of new glycolipid structures [9-11, 139-141]. Two glycolipids were isolated from the sponge *Pseudoceratina* sp.: a galactosyl diacylglyceryl, and an alkylacylglycerol linked to a five-membered cyclitol, with common fatty acyl chains ranged from 14:0 to 18:0 [142]. Ectyoceramide, the first natural hexofuranosylceramide has been isolated from *Ectyoplasia ferox* in a pure form, instead of a complex mixture as is usually found [11]. The FA attached to the amino group is a 2-hydroxylated *anteiso*-16:0. It has been demonstrated that a change in the stereochemistry of the glycosidic linkage from the usual β to the quite rare α configuration can affect their biological activity. Thus, α -glycosyl GSL, such as the natural Agelasphines and their synthetic derivative KRN7000 [143-145], are immunomodulating and antitumor compounds. Not all sponge glycolipids are GSL and a great variety of structures is observed.

These compounds stimulated the microtubule polymerization at 10 °C. The investigation of glycolipids from the Caribbean sponges allowed the isolation of Plakosides A and B from *Plakortis simplex* [10] (Fig. 15), and Plakosides C and D from *Ectyoplasia ferox* [140] (Fig. 15). These glycolipids are among the most fascinating lipids isolated from marine organisms. They have a unique structure with a prenylated galactose and two cyclopropanyl alkyl chains. The Plakosides had the same α -hydroxylated C₂₂ fatty acid amide^d with a cyclopropane at the C11-C12 positions. As the sponges are taxonomically distant, it is possible that these unusual glycolipids may originate from bacteria. Plakosides A and B are immunosuppressants that act through a non-cytotoxic mechanism [9, 10].

Crasserides, and their minor associated compounds isocrasserides, are widely distributed in marine sponges, and are considered to be glycolipids although their sugar unit is replaced by an unusual five-membered cyclitol [146] (Fig. 16).

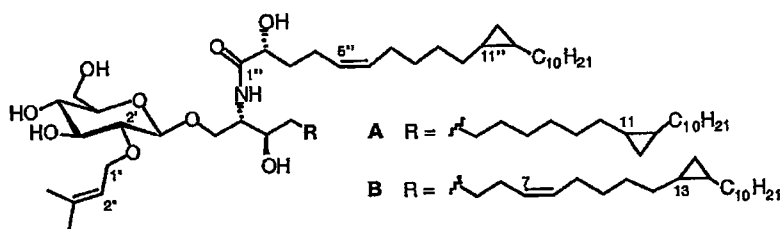
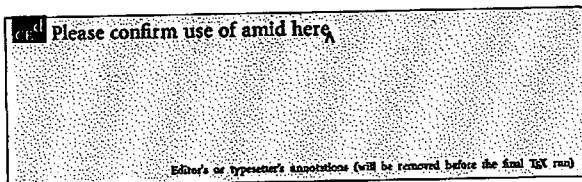


Fig. 15 Plakosides A and B from *Plakortis simplex*, and Plakosides C and D from *Ectoplasia ferox*



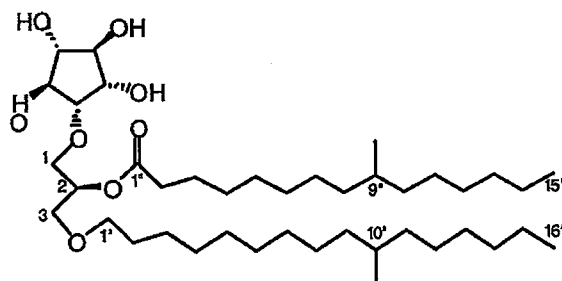


Fig. 16 An example of an isocrasseride isolated from *Plakortis simplex*

The cyclitol is linked to the glycerol with an O-3 ether bond. Also linked to the glycerol are an O-1 alkyl group and an O-2 acyl group. Furthermore, various fatty acyl chains are present including mid-chain branched chains. The Italian group has found crasserides in all sponges whose glycolipids have been studied.

8.2

Coelenterate – Cnidaria

Representatives of the Coelenterate phylum have remarkable peculiarities in their FA composition. Some species contain unusual FA or unusual concentrations of common components. Thus, large amounts of tetracosapolyenoic acids, namely 24:6(n-3) and 24:5(n-6), were found in different orders of the Octacorallia subclass of Anthozoa [147]. Gorgonian corals are of interest since some of these invertebrates are known sources of methylene-interrupted PUFA, in particular of the (n-3) and (n-6) series [148, 149]. The gorgonian specimens harvested in colder waters contained high amounts of methylene-interrupted PUFA, unlike specimens from warmer waters. This could have been due to the temperature or to the high content of wax esters. Nevertheless, arachidonic acid, a major component in all FA mixtures studied (14–21%), is a well-known precursor of prostanoid compounds. The high levels of tetracosapolyenoic FA in specimens from colder waters were of particular interest: 24:6(n-3) (5.1–5.3%), 24:5(n-6) (8.4–15.8%) and 24:5(n-3) (5.0–5.2%) [149]. An analysis of four gorgonians from the genus *Pseudopterogorgia* revealed that the main PUFA are 18:3(n-6), 18:4(n-3), AA, DHA, 24:5(n-6) and 24:6(n-3), with the (n-6) PUFA predominating [150]. All five gorgonians of the genus *Eunicea* presented a similar phospholipid FA composition with unsaturated acyl chains from C₁₈ to C₂₄, as shown below in Table 1 [151]. 2-Hydroxy long-chain acids also occurred in these gorgonians. Several 2-hydroxy FA have been identified before in marine sponges [152, 153].

Table 1 Principal unsaturated FA in phospholipid from gorgonians of the genus *Eunicea* [151]

Fatty acid	<i>Eunicea</i> sp.	<i>E. fusca</i>	<i>E. laciniata</i>	<i>E. mammosa</i>	<i>E. succinea</i>
18:4(n-3)	24.6	16.6	7.5	7.6	15.2
18:3(n-6)	18.0	15.8	17.0	9.8	10.2
18:2(n-6)	0.7	1.5	4.9	1.3	1.5
18:1(n-9)	4.2	9.6	2.4	1.0	2.2
20:5(n-3)	1.3	1.5	3.1	1.3	1.5
20:4(n-6)	12.4	12.4	13.5	15.7	11.5
22:6(n-3)	5.1	6.3	4.2	3.5	6.2
24:5(n-6)	3.8	1.5	2.5	14.2	3.9
24:6(n-3)	0.6	0.4	1.4	2.9	2.7

Several species also contained 2-OH-20:0 and 2-OH-22:0 acids up to 5% of total FA PL. All species contained the new (Z)-7-Me-16:1(n-10) and (E)-7-Me-16:1(n-10) (0.5–2%)

The phospholipid FA composition of the Caribbean gorgonians *Gorgonia mariae* and *Gorgonia ventalina* (Gorgoniidae) was investigated [154]. This study reports that the main FA were 14:0, 16:0, 18:3(n-6), 18:4(n-3), 18:2(n-6), AA, DHA and 24:5(n-6), as shown in Table 2. In both gorgonians (n-6) PUFA predominated over the (n-3) family. In addition, Table 2 gives data for other gorgonians of the family Gorgoniidae [150].

Table 2 Main phospholipid unsaturated fatty acids of gorgonians from the family Gorgoniidae

Fatty acids	<i>Gorgonia mariae</i>	<i>Gorgonia ventalina</i>	<i>Pseudopterogorgia</i> *
18:3(n-6)	10.6	15.8	7.3
18:4(n-3)	10.8	16.4	12.0
18:2(n-6)	8.6	10.3	4.6
18:1(n-9)	5.0	4.0	3.0
20:4(n-6)	10.0	9.4	17.2
20:5(n-3)	6.4	1.2	0.2
22:6(n-3)	5.6	8.6	6.1
22:4(n-6)	2.2	0.3	—
24:5(n-6)	4.0	1.4	10.2
24:6(n-3)	2.0	0.4	3.7

* average of 4 *Pseudopterogorgia* species

(Z)-7-Me-16:1 n-10 and (E)-7-Me-16:1 n-10 occurred at 0.8–3.5%

2-OH-21:0 and 22:0 acids were identified at < 1%

Within the experimental errors, all of these gorgonians share a similar phospholipid FA profile, in as much as: 1) they all biosynthesize, in a similar ratio, the acids 24:5(n-6) and 24:6(n-3), with the former predominating, 2) the (n-6) family of FA predominates over the (n-3) family, and 3) the polyunsaturated FA DHA and AA are key FA in these gorgonians. Several new branched unsaturated FA occurred in gorgonians [149–151]. The New Caledonian gorgonian *Rumphella aggregata* also contained 24:5(n-6) (11%), NMID FA, and unusual short branched-chain unsaturated acids [155].

An interesting investigation on the FA composition of solar coral *Heliopora coerulea* (Octacorallia, Helioporacea) supported a chemotaxonomic significance of tetracosapolyenoic acids in Coelenterates [148]. 24:6(n-3), a tetracosahexaenoic acid, was found in *Heliopora coerulea* [148]. The major FA were 16:0 (40–42%) and 18:3(n-3) (15–16%), as shown below.

Table 3 Main fatty acids from total lipids of *Heliopora coerulea* [147]

Fatty acids	% for two samples	Fatty acids	% for two samples
14:0	4.8–4.8	18:4(n-3)	3.5–3.6
16:1(n-7)	3.1–3.3	18:0	7.3–5.0
16:0	40.9–41.6	20:5(n-3)	5.4–5.5
18:1(n-9)	3.1–3.5	22:6(n-3)	4.7–5.5
18:3(n-6)	15.1–15.8	24:6(n-3)	1.7–1.9

Despite a relatively low content of 24:6(n-3) (2% of total FA), it was concluded that PUFA of regular structure with 24 carbon atoms and five to six methylene-interrupted cis-double bonds are typical constituents of representatives of all orders of the Octacorallia, Alcyonaria, Gorgonaria, Helioporida and Pennatularia. Three novel 10-hydroxydocosapolyenoic acids were isolated from deep-water scleratinian corals, as shown below [156].

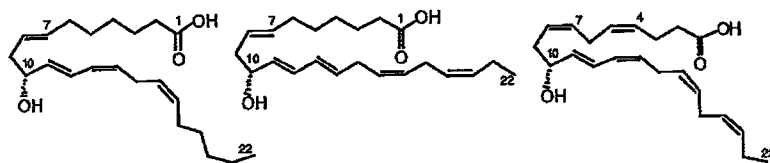


Fig. 17 10-Hydroxypolyenoic FA from *Madrepora oculata*

A work has recently been carried out aimed at elucidating the biosynthesis of docosahexaenoic acid in trout liver microsomes [157, 158]. This report concludes that the tetracosahexaenoic acids, such as 24:6(n-3), are intermediates in the biosynthesis of DHA. Therefore, it is very likely that gorgonians

utilize a similar biosynthetic route, thus providing yet another interesting system to study this type of biogenesis. However, work still remains to be done on the role of symbiotic zooxanthellae in the production of some of these unusual FA.

A survey of lipid and FA composition was made for 15 cnidarians from Okinawa, Japan [159]. Corals having symbiotic Zooxanthellae within their cells contain large amounts of lipid in their tissues (24–26% of dry weight). All specimens contained monoalkyldiacylglycerol and were rich in wax esters and triacylglycerol. Palmitic acid was the most abundant FA component of these lipid classes (more than 40% in each class), followed by stearic and oleic acids [159]. Four ceramides with the same hexadecyl acyl chain isolated from a gorgonian exhibited significant human cholesteryl ester transfer protein inhibitory activity [160].

8.3

Echinodermata

Significant amounts of tetracosapolyenoic acids such as 24:6(n-3) were found in different marine organisms, echinoderms (Ophiuroidea and Crinoidea) and coelenterates [147], and in their predators [161]. 24:6(n-3) occurred in symbiotic and non-symbiotic brittlestars [162]. Isolated from the brittlestar *Ophiura sarsi* [163], this FA had anti-inflammatory and anti-allergic properties similar to those of DHA [164]. Total lipids of this organism contained 14% of 24:6(n-3), 15% of EPA and 2.6% of DHA [163]. Furthermore, high levels of EPA and 24:6(n-3) were observed in phospholipids that accounted for more than 50% of total lipids from *O. sarsi*. Several novel NMI FA were further identified in *O. sarsi* [165], namely 7E,13E-20:2, 7E,13E,17Z-20:3 (13% of total FA), 9E,15E,19Z-22:3, and 4Z,9E,15E,19Z-22:4 acids, as shown below.

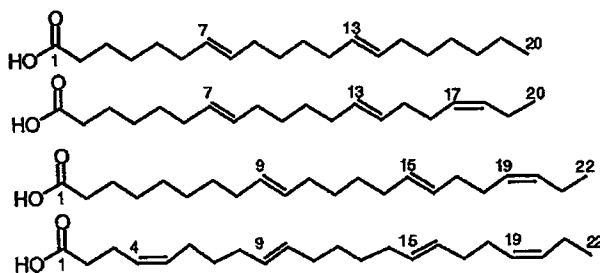


Fig. 18 New non-methylene-interrupted FA from *Ophiura sarsi*

Other NMI FA also occurred in this brittlestar, namely 5,11-20:2, 5,13-20:2, 7,13-22:2 and 7,15-22:2. 24:6(n-3) Accounted for about 6%. Structural analyses of these fatty acids were performed after partial hydrogenation

with hydrazine sulfate and subsequent isolation of the monoenoate products by argentation thin-layer chromatography, followed by GC/MS analyses of dimethyl disulfide adducts [165]. A comparison of FA in symbiotic and non-symbiotic brittlestars from Oban Bay, Scotland, was performed in order to look for bacterial signals that might indicate contribution of subcuticular bacteria to their hosts' diet [162]. Odd-chained and branched FA were present in low amounts but palmitoleic and cis-vaccenic acids, also considered as good markers for bacteria, occurred at much higher amounts in symbiotic species studied than in the non-symbiotic species. EPA occurred at high levels in all species studied (11–23%) but DHA was present at low amounts. The unusually high concentrations of 24:6(n-3) (up to 15%) may indicate that DHA derived from dietary phytoplankton is being elongated to the former [162]. Furthermore, three other tetracosapolyenoic acids and 26:6(n-3) were found in some species [162].

Three new ceramides from the starfish *Acanthaster planci* have been reported [166]. Several glycosphingolipids have been identified recently in sea cucumbers possessing α -hydroxylated or non-hydroxylated, saturated or monoenoic fatty acyl chains [167–169].

8.4

Tunicates

Few works have been published on the lipid composition of tunicates [147, 157, 170, 171]. Lipids of edible ascidian *Halocynthia roretzi*, very popular in Japan and Korea, have also been studied [170]. Several studies of phospholipids of pelagic tunicates (belonging to gelatinous zooplankton) have also been undertaken [171]. PUFA represent the most important class, accounting for around 50%. The tunicates *Eudistoma bituminis* and *Cystodytes violatinctus* from the Indian Ocean were investigated for their phospholipid FA content [172]. In both cases, the most abundant FA were the saturated ones (C₁₀ to C₁₈). *Cystodytes violatinctus* contained high amounts of oleic acid (20%). Both *E. bituminis* and *C. violatinctus* contained phytanic acid and Δ 10 FA, which had not previously been found in such organisms. These tunicates contained only trace amounts of PUFA, which are usually predominant in this phylum [172].

8.5

Molluscs

8.5.1

Introduction

Lipids are a very important food reserve, in particular in the oocytes of molluscs, which assures viability of the larvae. Lipids also provide energy for

growth during conditions of limited food supply when carbohydrate levels (the main energetic reserve in molluscs) are low. Phytoplankton represent the largest food source for bivalve molluscs and contain a high proportion of PUFA. The intensive rearing of bivalves still relies on the massive production of unicellular algae especially for growing young spat, which represent the largest biomass in a commercial hatchery. The high cost and unpredictable culture success of algae has inspired the development of artificial diets such as microcapsules, mixed diets, yeast based diets, lipid microspheres and liposomes to substitute or supplement live algal diets. The importance of lipids in bivalve nutrition is now well known [173]. The (n-3) PUFA, EPA and DHA, have been reported to be essential for optimal growth for at least some species of juvenile bivalves.

The biochemical composition of the intertidal rocky-shore bivalves, e.g. mussels, is greatly affected by periods of air exposure, at which times the bivalves are denied a food source. Consequently, the resulting effect would be similar to starvation [174].

In coastal environments, detritus, bacteria and zooplankton may greatly affect available food composition. It is known that organic detritus areas an energy source for bivalves during periods of scarce primary productivity. Additionally, detrital material is a source of saturated and monounsaturated C₁₄-C₁₈ FA. A high proportion of SFA, such as 20:0, has been observed in bivalves distributed in environments rich in organic material with an abundant bacterial load [175], compared with those mainly nourished by marine phytoplankton, which are dominated by (n-3) PUFA of 18, 20 and 22 carbons. The lipid composition of molluscs can be affected by external factors, such as fluctuations in the environmental conditions and qualitative and or quantitative changes in food availability, or by internal factors, such as sexual maturation [175]. Recently, some very interesting investigations have dealt with the chemical composition and chemotaxonomic of cardiolipids from some marine bivalves and led to evidence of a tetradocosahexaenoic cardiolipin [176].

8.5.2

Mussels

FA profiles of seeds of the mussel *Mytilus galloprovincialis* originating from two habitats (rocky shore and subtidal) were compared after transfer to the same habitat (subtidal), in order to study the initial levels of different FA of metabolic importance and their variability [177]. According to previous studies, PUFA were found to be the group with highest percentage (42-49% of total FA), including EPA and DHA as major components [177]. Moreover, these findings concur with recent studies of numerous bivalve species distributed in other regions of Europe and America, such as *Argopecten purpuratus* [178] and *Crassostrea gigas* [179]. Additionally, the mussels of subtidal origin presented higher initial levels than the rocky-shore mussels with re-

gard to FA characterized by energetic-type functions, such as 14:0, 16:0 and EPA. High initial levels of some PUFA observed in the subtidal mussels, such as 18:3(n-3), 18:4(n-3) and EPA, are presumably due to the fact that these mussels had greater access to phytoplanktonic food. Initial levels of 14:0, 16:0 and 18:0 in the rocky-shore mussels were significantly greater than in the mussels of subtidal origin. FA characterized by structural-type functions, e.g. 18:0, DHA and NMID FA with 20 and 22 carbons, in rocky-shore mussels presented higher levels than those of the subtidal mussels. These NMID FA have been observed in greater proportions in phospholipids, thus implying a structural-type function [177].

In addition, it is also known that these NMI FA are distributed in greater quantities in the organs more exposed to the immediate environment, such as the gills, mantle and foot. In another study, minor NMI FA were characterized in *Mytilus galloprovincialis* by GC/MS of their 2-alkenyl-4,4-dimethyloxazoline derivatives, namely 7,13-16:2, 5,11-20:2, 5,13-20:2, 7,15-22:2, and the new trienoic FA 5,11,14-20:3 and 7,13,16-22:3 [180]. This discovery supports a biosynthetic route implying the desaturation and subsequent elongation of 20:2(n-6) [180]. Lipid, FA and sterol composition of the New Zealand green-lipped mussel (*Perna canaliculus*) and the Tasmanian blue mussel (*Mytilus edulis*) have also been reported [181].

8.5.3

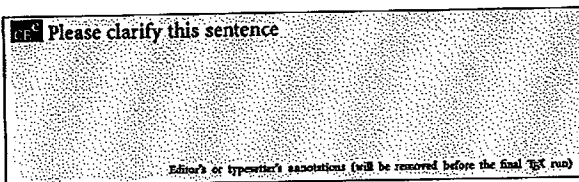
Oysters

Seasonal variations of lipid classes and FA in the flat oyster *Ostrea edulis* have been studied [182]. The dynamics of FA in the larval development, metamorphosis and post-metamorphosis of this oyster have been investigated [183]. The lipid composition of *Crassostrea gigas* was analysed during the reproductive phase in natural as well as under artificial conditions [179]. A specific accumulation of DHA and EPA in the polar lipids was observed under both conditioning diets. The proportions of DHA and EPA from neutral and polar lipids of oysters conditioned artificially were significantly lower than of those that were naturally conditioned. A useful comparison of the lipid class and FA composition between a reproductive cycle in nature and a standard hatchery conditioning of the Pacific oyster *Crassostrea gigas* was performed [179].

8.5.4

Patella

The effects of season and spatial distribution on the FA composition of four *Patella* species' gonads and soft body tissue were evaluated [185]. The results show that the quantitatively most important FA were 14:0, 16:0 and 18:0; the MUFA 18:1(n-7), 18:1(n-9), 16:1(n-7) and 20:1(n-9), EPA and AA. *P. depressa* and *P. ulysiponensis* soft-body FA profiles revealed significant differences be-



tween sexes, with males showing significantly higher percentages of PUFA, mainly EPA and AA, while in females significantly higher proportions of MUFA were found. The fatty-acid composition of *P. depressa* gonads revealed significant differences between sexes, [185] which were more acids from the (n-3) and (n-6) series (AA and EPA), while females were seen to have higher proportions of SFA [185].

8.5.5

Clams

Recent studies on clam lipids were concerned with their aquaculture. Thus, the possible use of emulsions rich in EPA and DHA as an artificial lipid supplement to live algae was investigated for seed of the Manila clam *Tapes philippinarum* [173]. In addition, the influence of the lipid composition of microalgal diets and cornstarch on the lipid classes and FA of the *Ruditapes decussatus* spat was studied [186]. This clam species is of commercial interest in Spanish aquaculture. In order to increase its production, experiments have been carried out with alternative foods to live microalgae, such as freeze-dried microalgae or cornstarch. The main FA present in the spat of *R. decussatus* were 16:0, 18:1(n-9) and DHA, followed by lower contents of 18:4(n-3) and 18:1(n-7). The essential FA EPA is present in small amounts. The content of (n-3) PUFA, (n-9), (n-6), and 20:2 and 22:2 NMID FA differed significantly according to the diet supplied. Spat fed on a microalgal diet show the significantly highest content in (n-3) PUFA and (n-9) FA [186].

8.5.6

Scallops

Overfishing has often resulted in a decline of natural beds of *Pecten maximus* in several areas. Thus, several experiments have been performed on *P. maximus* culture, growth and reproduction to improve the biological knowledge of this species [187, 188]. Lipids are a very important food reserve in the oocytes, which assures viability of the larvae. It has been shown that success of *Pecten maximus* hatching is related to the lipid status of the eggs when spawned. Lipids in the female gonad were analysed for lipid class composition and FA composition of TAG and phospholipids, and their variations in relation to gametogenic cycle were studied [184–188]. PUFA were more abundant in the TAG and the series (n-3) was clearly predominant. The principal (n-3) FA (mainly in phospholipids) showed a seasonal variation clearly related to the reproductive cycle [188]. Soudant et al [184] have analysed the composition of polar lipid classes in male gonads of *Pecten maximus* and the effect of nutrition. Seasonal digestive-gland dynamics of the scallop *Pecten maximus* have been reported [189].

The distribution of lipids and FA in different organs of the Chilean scallop *Argopecten purpuratus* broodstock, namely female and male gonads, digestive gland, gills, mantle and adductor muscle, have been investigated [178]. The highest level of (n-3) PUFA (mainly EPA and DHA) was found in the adductor. The major FA in all parts studied were 16:0, EPA (8.1–20.3%) and DHA 9.2–25.6%. Similarities between the FA composition of the triglyceride fraction of the female gonad and the digestive gland (e.g. the high level of 14:0 and 18:4(n-3)) indicated the transfer of lipids from the lipid-rich digestive gland to the female gonad. A special feature of the gills and mantle was the presence of high levels of plasmalogens (phosphoglycerides with 1-alkenyl chains) recognized by the presence of dimethylacetals, which are formed simultaneously with FAME by acid-catalyzed transmethylation [178]. In another investigation, dietary supplementation with lipid emulsions during broodstock conditioning of *A. purpuratus* was used to manipulate the fatty-acid composition of the eggs [187, 190]. The scallops were fed a mixed algal diet either alone or supplemented with an emulsion rich in ethyl esters of DHA or EPA. Lipid supplementation resulted in a significant increase of the total lipid content of the eggs. The EPA and DHA levels in the total and neutral lipids of eggs from broodstock supplemented with diets including the corresponding emulsions were significantly higher than in eggs from scallops fed solely algae [190, 191]. NMID FA, which are not present in the phytoplankton, have been reported in many species of molluscs. Thus, they are presumably synthesized by molluscs. Interestingly, the identification and occurrence of a novel *cis*-4,7,10,trans-13-docosatetraenoic acid in the female gonads of the scallop *Pecten maximus* was described [192].

Lipid deterioration during frozen storage at -20°C of the adductor muscle, the major edible part of the giant scallop, was examined by determination of fatty chain compositions in the sn-1 and/or sn-2 positions of ether and ester glycerophospholipids [193]. During storage, the contents of total lipid and polar lipid decreased but that of non-polar lipid increased. The percentages of PUFA such as EPA and DHA in the total lipid and polar lipid fractions decreased during storage, but those of the PUFA in the non-polar lipid increased. Changes during storage in alkenyl and alkyl chain compositions of ether glycerophospholipids and in fatty acyl chain compositions of ether glycerophospholipids were determined [193]. The 20:2 and 22:2 NMID FA also occurred.

8.5.7

Squids

Biochemical composition changes and FA in several tissues of the squid *Illex argentinus* from the South Atlantic Ocean at different sexual stages were studied. All tissues contained high concentrations of PUFA followed by SFA and MUFA, e.g. 16:0, 18:0, 18:1(n-9), 20:1, 22:1, EPA and DHA. The lipids

also contained unusually high levels of FA of the linoleic family (2–3%). The digestive gland of the squid is rich in polyenoic FA, EPA and DHA. This tissue could be a cheap raw material, presently discarded, for production of PUPA [194].

The lipid content and composition of phosphatidylcholine and FA were determined in tissues of 70 species of teleosts, and in muscle of six species of squid. Amount and composition of diacyl glyceryl ethers were recently analysed in various tissue lipids of the deep-sea squid *Berryteuthis magister* [195].

9

Crustacea

The mud crab *Scylla serrata* is a commercially important species in the Indo-Pacific region and has been cited as a target species for a stock-enhancement program in Japan and also as a target species for aquaculture in many Asian countries [196]. A recent study evaluated the requirements of linoleic acid (18:2(n-6)), linolenic acid (18:3(n-3)), EPA and DHA during rotifer, as well as *Artemia* feeding on survival and larval development of mud-crab larvae [196]. Decreased natural seed availability and the low survival rate in crab hatcheries [197–199] have been major problems in increasing aquaculture production. Moreover, Takeuchi et al [197] described the requirement of EPA and DHA for larval development, where EPA is effective in maintaining survival while DHA plays an important role in accelerating the intermolt period and produces a wider carapace width in swimming crab larvae. A previous study showed that the *Artemia* feeding schedule (in combination with rotifers) for larval mud crab and their essential FA composition affected the survival of larvae [199–201].

If the conditions of thermal adaptation are well documented in fish, little information is available for marine invertebrates. A comparative study of the phospholipid FA compositions was conducted with the Baltic Sea amphipod crustacean *Gammarus* spp. collected from different thermal environments [202]. It was reported that environmental temperature had little effect on FA composition. In fact, *Gammarus* was shown to use the same strategy to control membrane fluidity in the cold as fish species investigated so far [203], namely the crustacean accumulates sn-1 monoenoic and sn-2 polyenoic phospholipid FA at reduced temperatures.

10

Polyunsaturated FA (n-3) of commercial interest

10.1

Introduction

An "essential" nutrient is one that is needed for normal development and function of mammalian cells throughout the life cycle. In its active or precursor form there is a minimum amount of such a nutrient that must regularly be provided in the diet. This dietary requirement generally varies with species, gender, age and the presence of physiological and pathological challenges (pregnancy, lactation, infancy, aging, infection, disease, etc.).

The term "essential fatty acid" is ambiguous and inappropriately inclusive or exclusive of many polyunsaturated FA. When applied most rigidly to linoleate and a linolenate, this term excludes the now well-accepted but conditional dietary need for two long-chain polyunsaturates (arachidonate and docosahexaenoate) during infancy. The metabolism of essential and nonessential FA is clearly much more interconnected than previously understood. Replacing the term "essential fatty acid" by existing but less-biased terminology, i.e. polyunsaturates, (n-3) or (n-6) polyunsaturates, or naming the individual fatty acid(s) in question, would improve clarity and would potentially promote broader exploration of the functional and health attributes of polyunsaturated FA [204].

10.2

Health benefits

Initially, it should be noted that the chemical form of the PUFA supplementation used for clinical assays and up to the final consumers will not be detailed. Indeed, sometimes they take TAG forms while occasionally they are ethyl esters or free fatty acids, depending on the target.

Polyunsaturated FA (PUFA) are essential components in higher eukaryotes that confer fluidity, flexibility and selective permeability to cellular membranes. PUFA affect many cellular and physiological processes in both plants and animals, including cold adaptation and survival [205, 206], modulation of ion channels [207, 208], endocytosis/exocytosis [209], pollen formation, pathogen defense, chloroplast development in plants [210], and activities of membrane-associated enzymes that are sensitive to the biophysical properties of lipid membranes [211–213].

In mammals, metabolism of LC-PUFA by oxygenases yields a range of important short-lived molecules (generically known as the eicosanoids), such as prostaglandins, leukotrienes and thromboxanes (Fig. 19). These resulting metabolites bind to specific G-protein-coupled receptors and signal cellu-

lar responses and modulate many biological processes (see below). Because the production of various classes of these molecules depends in part upon the availability of their PUFA precursors in membrane phospholipids, modulation of PUFA is a potential target of pharmaceuticals and nutraceuticals [213,214].

The (n-3) LC-PUFA, particularly EPA and DHA, are thought to display a variety of beneficial effects in areas ranging from foetal development to cancer prevention [215]. Some of those health effects are presented below.

10.2.1

Heart health

It is well established that populations with a high consumption of oily fish have a lower incidence of heart disease and several studies have confirmed that EPA and DHA are the protective components [216–224].

Recent research concludes that perhaps the most important effect of (n-3) LC-PUFA, when it comes to preventing cardiovascular disease, is their ability to stabilize atherosclerotic plaque by reducing the infiltration of inflammatory and immune cells (lymphocytes and macrophages) into the plaque [225]. This could explain the reduction in fatal and nonfatal heart attacks and strokes associated with an increased intake of fish oils [226]. In addition, the anti-inflammatory effect of DHA by reducing the C-reactive protein (CRP) level in blood may decrease the risk of coronary heart disease such as atherosclerosis [227].

Several large clinical trials have confirmed the ability of fish oils to prevent sudden cardiac death in both presumably healthy subjects as well as in patients having suffered a heart attack [228–231]. Although most research so far has focused on the effect of (n-3) on life-threatening ventricular arrhythmias it is likely that many of the findings may also be applicable to atrial fibrillation [232]. It has also been shown that a high (n-3) content of blood cells and serum cholesterol esters is associated with increased heart rate variability and leads to a decreased risk of cardiac disease and a longer lifespan [233]. In addition, EPA and DHA seem to reduce the mortality among not only patients who have survived a first heart attack [234–236] but also among old people [237,238].

Numerous studies have confirmed that (n-3) LC-PUFA included in fish oils significantly combat hypertension by a notable reduction of blood pressure and benefit heart transplant patients [239–247].

EPA and DHA are also effective in lowering the blood level of triglycerides [248,249], in improving large artery dilation in patients with high cholesterol levels [250], and they possess antithrombotic effect [251]. Such positive actions also contribute to reduce the risk of cardiovascular disease.

10.2.2

Cancer

Several studies have shown the effect of LC-PUFA against cancer such as an inverse relationship between blood levels of EPA and DHA and the risk of prostate cancer [252, 253] or fish and fish oil consumption and adenocarcinomas [254]. In addition, (n-3) PUFA can also act positively against cancer effects like cachexia (abnormal weight loss) or survival rate in end-stage cancer [255, 256].

10.2.3

Arthritis

The (n-3) PUFA are also known to decrease rheumatoid arthritis; notably by lowering interleukin-1 β production which results in a significant reduction in morning stiffness and the number of painful joints [257–261].

10.2.4

Psoriasis

Psoriasis is a fairly common skin disease characterized by high concentrations of AA in the plaques and profound changes in the metabolism of eicosanoids leading to an increase in proinflammatory agents. Some studies have shown that n 3 PUFA, notably EPA, can counteract the formation of these proinflammatory agents and that oral supplementation with fish oils benefit psoriasis patients [262, 263].

10.2.5

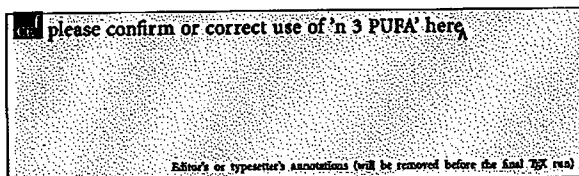
Lung disease

A few years ago it was shown that children who regularly eat fresh, oily fish have a four times lower risk of developing asthma than children who rarely eat such fish. EPA was suspected to be responsible by reducing airway inflammation and responsiveness. Later, studies on supplementation by (n-3) LC-PUFA have confirmed their benefit in the reduction of breathing difficulties and other symptoms in asthma patients. More recently, it has been demonstrated that those PUFA are also beneficial in the treatment of other lung diseases such as cystic fibrosis and emphysema [264–267].

10.2.6

Attention-deficit disorder

Attention-deficit hyperactivity disorder (ADHD) is characterized by hyperactivity, emotional instability, poor coordination, short attention span, poor



concentration, impulsiveness, and learning disorders. It is very common among school-age children with an incidence of 4–20%. Initial studies have linked ADHD to a deficiency of certain long-chain FA, notably AA, EPA and DHA [268]. More recently Burgess et al. [269] have found that children with ADHD were breastfed less often as infants than children without ADHD (breast milk is an excellent source of DHA). In addition to ADHD, other disorders such as dyslexia (difficulties in learning to read and write) and dyspraxia (problems with coordination and muscle control) also have deficiency in LC-PUFA as a common denominator that may be avoided by oral supplementation of concentrated fish oils [270].

10.2.7

Mental health

Several epidemiological studies have shown that a high dietary intake of linoleic acid and a low intake of EPA and DHA are associated with cognitive impairment and an increased risk of dementia. In a recent study, it was that EPA and especially DHA help keep the membranes of brain cells more fluid while saturated and (n-6) FA tend to "harden" them. Authors believe this and the anti-inflammatory effects of EPA and DHA are what help preserve cognitive function [271].

In addition to preventing dementia, (n-3) PUFA help in combating depression, schizophrenia, Alzheimer's disease and other mental illnesses [272–283].

10.2.8

Pregnancy and infancy

An adequate intake of DHA and EPA is particularly important during pregnancy and lactation. During this time the mother must supply all the baby's needs for DHA and EPA because it is unable to synthesize these essential FA itself. DHA makes up 15–20% of the cerebral cortex (a normal adult human brain contains more than 20 g of DHA) and 30–60% of the retina (it is also concentrated in the testes and sperm), so it is absolutely necessary for normal development of the foetus and baby, which implies optimal levels in pregnant and lactating mothers. There is some evidence that an insufficient intake of (n-3) FA may increase the risk of premature birth and an abnormally low birth weight. There is also emerging evidence that low levels of (n-3) acids are associated with hyperactivity in children [268, 284–292].

The constant drain on the mother's DHA reserves can easily lead to a deficiency that may be linked to preeclampsia (pregnancy-related high blood pressure) and postpartum depression [288–290].

10.3

Nutrition: Importance of the ratio of (n-6) and (n-3) essential FA

There are good fats and there are bad fats. Artificially produced *trans*-FA are bad in any amount and saturated fats from animal products should be kept to a minimum. The best fats, or oils rather since they are liquid at room temperature, are those that contain the essential FA that are so named because without them we die (see chapter introduction). Essential FA are polyunsaturated and grouped into two families, the (n-6) EPA and the (n-3) EPA.

Seemingly minor differences in their molecular structure make the two EPA families act very differently in the body. 18:2(n-6) and 18:3(n-3) are not interconvertible and compete for the rate-limiting 6-desaturase in the synthesis of LC-PUFA (see biosynthesis). AA and EPA are the parent compounds for the production of eicosanoids with opposite properties, see Fig. 19. Many scientists believe that a major reason for many diseases (see below) is the profound imbalance between our intake of (n-6) and (n-3) FA. Our ancestors evolved on a diet with a ratio (n-6)/(n-3) of about 1 : 1. A massive change in dietary habits over the last few centuries (modern agriculture) has changed this ratio to something closer to 20:1¹ [293–295].

An increase in the dietary intake of (n-6) EPA changes the physiological state to a prothrombotic, proconstrictive, and proinflammatory state. Many of the chronic conditions, cardiovascular disease, diabetes, cancer, obesity, autoimmune diseases, rheumatoid arthritis, asthma and depression, are associated with increased production of thromboxane A₂ (TXA₂), leukotriene B₄ (LTB₄), IL-1 β , IL-6, tumor necrosis factor (TNF), and C-reactive protein [295]. All these factors increase with (n-6) fatty-acid intake and decrease with (n-3) fatty-acid intake, whether 18:3(n-3), 20:5(n-3) or 22:6(n-3). EPA and DHA are more potent², and most studies have been carried out using EPA and DHA (see above).

The optimal dose or ratio of (n-6)/(n-3) varies from 1/1 to 4/1 depending on the disease under consideration. In the secondary prevention of cardiovascular disease, a ratio of 4/1 was associated with a 70% decrease in total mortality [296]. A ratio of 2.5/1 reduced rectal cell proliferation in patients with colorectal cancer, whereas a ratio of 4/1 with the same amount of (n-3) PUFA had no effect [297]. The lower (n-6)/(n-3) ratio in women with breast cancer was associated with decreased risk [298]. A ratio of 2–3/1 suppressed inflammation in patients with rheumatoid arthritis, and a ratio of 5/1 had a beneficial effect on patients with asthma, whereas a ratio of 10/1 had adverse consequences [265, 299]. These studies indicate that the optimal ratio may vary with the disease under consideration. This is consistent with the fact

¹ Lipid consumption in Europe nowadays: vegetal (65%) > land animals (17%) > butter (16%) > marine animals (2%).

² Alpha-linolenic acid can be converted to EPA and DHA in the body, but the conversion is quite inefficient, especially in older people [294].

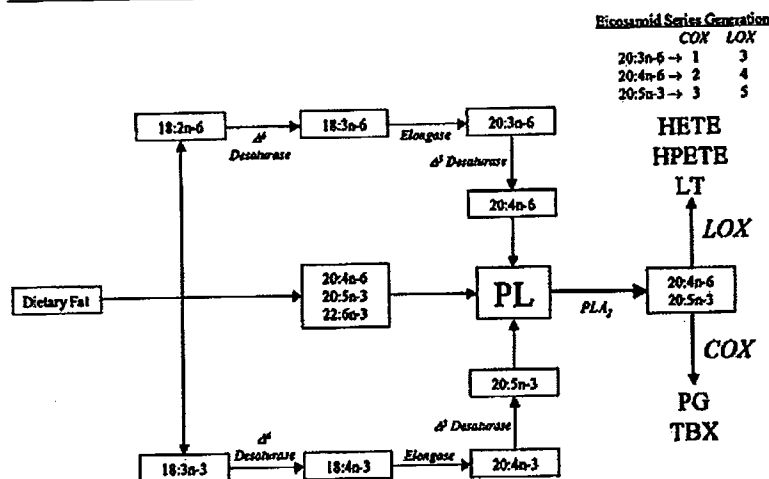


Fig. 19 Biosynthesis of eicosanoids from the essential FA [304]. Abbreviations: PL, phospholipids; PLA₂, phospholipase A₂; COX, cyclooxygenase; LOX, lipoxygenase; PG, prostaglandins; TBX, thromboxanes; HETE, hydroxy-eicosatetraenoic acids; HPETE, hydroperoxyeicosatetraenoic acids; LT, leukotrienes. Dietary lipids provide EFA and pre-formed substrates for the COX/LOX pathways. Dietary FA such as 20:3(n-6), 20:4(n-6), and 20:5(n-3) are direct precursors, while 18:2(n-6) and 18:3(n-3) must be elongated and desaturated prior to their conversions to eicosanoids

that chronic diseases are multigenic and multifactorial. Therefore it appears important to restore the balance between (n-6) and (n-3) for homeostasis and normal development. On this basis and by recognizing the unique benefits of EPA and DHA and the serious consequences of a deficiency, recommendations for daily intake of (n-3) PUFA has been published by several international scientific authorities [300–303].

10.4

Routes for biosynthesis

Despite extensive screenings, no angiosperm plants have been detected that accumulate in reserve triacylglycerols or in membrane lipids cis-polyunsaturated FA with carbon chains longer than C18 [305]. However, it is well known that polyunsaturated FA have crucial roles in membrane biology and signalling processes in most living organisms (see the section on health benefits). As indicated by Abbadi et al. [306], the daily requirement of such PUFA may vary and depend on the availability of linoleic and linolenic acid for conversion by elongation and desaturation, but a more regular consumption and an accordingly sustainable source of PUFA would be highly

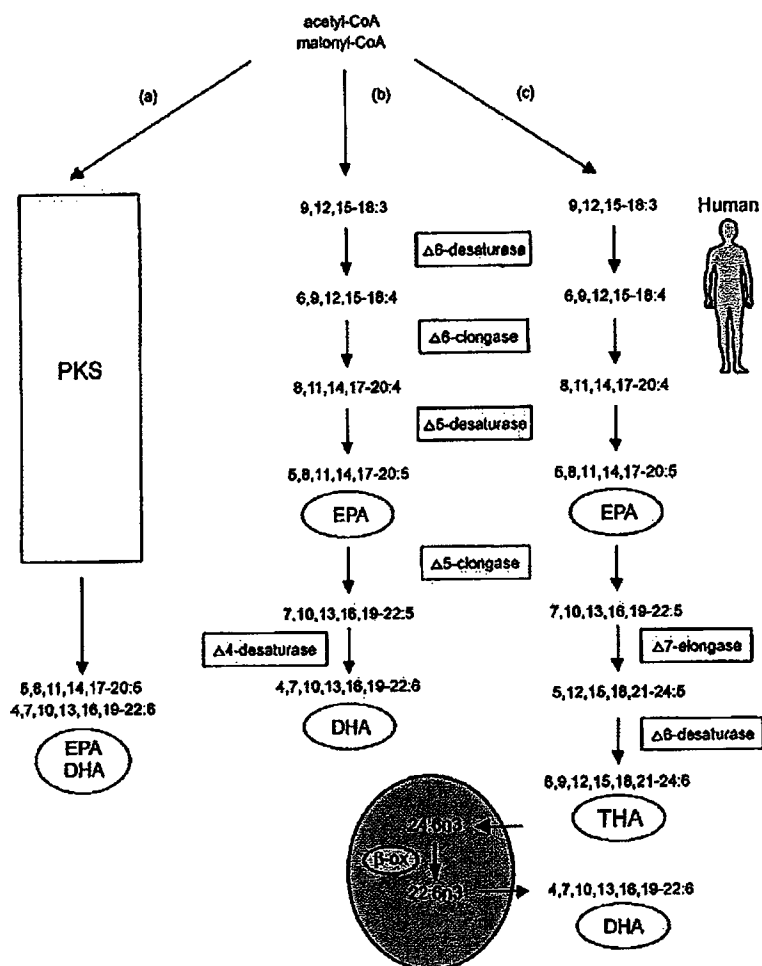


Fig. 20 Pathways of very-long-chain polyunsaturated synthesis in different organisms. Major products are indicated in ovals while enzymes are boxed. The anaerobic route (a) makes use of a polyketide-like system (PKS), whereas aerobic routes (b, c) use different enzymes (desaturases and elongases). If routes (b, c) are started with linoleic acid (9,12-18:2), arachidonic acid (AA) = 5,8,11,14-20:4 is obtained. Route (b) is a direct pathway that may operate in marine primary producers and initiate the food chain of "oceanic" polyunsaturated FA ending in large carnivorous fish. Route (c) represents the Sprecher [22] pathway as typical for mammalian cells. Mammals lack Δ12 and n3 desaturase activities and obtain linoleic acid and α-linolenic acid (9,12,15:3) from their diets. In this route, the synthesis of DHA is now known to consist of two succeeding elongation cycles, a Δ6 desaturation and a β-oxidation chain-shortening

desirable. At present, these FA enter the human diet mainly in the form of marine and freshwater fish. But in view of the increasing human population, the overfishing of marine resources, the dependence of fish farming on PUFA from fish oil and the environmental impact of aquaculture systems, neither farmed nor captured fish can be considered as a sustainable source of PUFA (see section on market) [307]. Thus alternatives have to be found such as new sources or by modifying existing ones by genetic approaches. For example, transgenic oilseeds could be a way out of the forthcoming shortage, particularly in view of the fact that a low percentage of PUFA in daily-consumed plant oils would satisfy nutritional requirements [306].

So, over the last few years, the biosynthetic LC-PUFA pathway has been the subject of much interest and it is only recently that molecular genetic approaches have allowed detailed studies of the enzymes involved in their synthesis. Thus, at this time, three distinct routes for LC-PUFA biosynthesis have been identified; two are aerobic and one is anaerobic (Fig. 20).

10.4.1

Aerobic pathways

In these routes, LC-PUFA biosynthesis is catalysed by sequential desaturation and fatty acyl elongation reactions. Known pathways involve the processing of the saturated 16:0 or 18:0 products of fatty acid synthase (FA) by elongation and aerobic desaturation reactions. The desaturase enzymes insert double bonds at specific carbon atoms in the fatty acid chain and the fatty acid elongation system elongates the precursors in two-carbon increments [308, 309]. Significant progress has been made in the identification of the enzymes required for PUFA synthesis; in particular, the fatty acid desaturases which are central to this pathway have now all been identified [308, 310]. The most relevant desaturases required for PUFA biosynthesis are the so-called "front-end" desaturases [311] that introduce a new double bond between an existing one and the carboxyl end of the acyl group. These "front-end" desaturases are all members of the cytochrome *b*₅ fusion desaturase superfamily, since they contain an N-terminal domain that is orthologous to the microsomal cytochrome *b*₅ [312].

Interestingly, there is a division of labor in higher eukaryotes for the synthesis of 20:4(n-6), 20:5(n-3) and other PUFA. Angiosperm plants convert oleic acid (18:1) to linoleic acid (18:2) and linolenic acid (18:3(n-3)), which are essential FA for mammals as substrates for the synthesis of C20, but these plants are unable to elongate the FA further. Mammals also convert 18:0 to 18:1(n-9) using a membrane-bound 18:0-CoA desaturase, however, they lack both $\Delta 12$ and (n-3) desaturase activities. Therefore, they require 18:2(n-6) and 18:3(n-3), the essential FA (EFA), in their diet [213]. These EFA are converted to long-chain PUFA via a series of desaturation and elongation reactions in the ER [308]. The $\Delta 6$ desaturase uses 18:2(n-6) and 18:3(n-3)

CTE please define abbreviation ER used here for the first time

as a substrates and inserts a double bond to produce 18:3 ω 6 and 18:4 ω 3. PUFA with a double bond at Δ 6 are substrates for the elongation machinery, which uses malonyl-CoA to add two carbons to the C-terminal end of the FA, producing 20:3(n-6) and 20:4(n-3). These FA are substrates for a Δ 5 desaturase, which produces 20:4(n-6) and 20:5(n-3) [213]. Those pathways for the synthesis of arachidonic acid (AA) and eicosapentaenoic acid (EPA) have been characterized biochemically and are supported by the recent cloning and characterization of desaturase and elongase genes.

In addition, some notable variations exist such as, for example, the free-living nematode *Caenorhabditis elegans* [313–316], the fungus *Mortierella alpina* [317] the moss *Physcomitrella patens* [318] the red algae *Porphyridium cruentum* [319], the freshwater protist *Euglena* [320] and the microalga *Isochrysis galbana* [321]. All these organisms possess particular enzymatic pathways.

At this stage of LC-PUFA biosynthesis a split occurs. The force of logic suggested that the steps succeeding AA and EPA synthesis would be another two-carbon elongation step and countervailing desaturation by Δ 4 desaturase to produce the C22 PUFA (elongation of EPA and AA to C22:5(n-3) and DTA, respectively, and the desaturation of the latter to form DHA and DPA). The cloning of a Δ 4 desaturase from the DHA-producing marine protist, *Thraustochytrium* sp., suggests that this pathway (route b, Fig. 22) is valid in some organisms [322]. However, mammals lack Δ 4 desaturase activity, and evidence has accumulated for an alternative pathway for C22 PUFA biosynthesis.

The sequence involving Δ 4 desaturase is the simplest one. It operates in various unicellular, eucaryotic algae belonging to different systematic groups and contributing to marine primary production. These and other photosynthetically active organisms are considered to be the primary sources of EPA and DHA entering marine feeding webs with large carnivorous fish and finally humans at the end [306, 307].

The other route, the so-called Sprecher pathway (route c, Fig. 20), remained controversial but recent experiments have indicated that it is the predominant route to DHA in mammals [22, 23, 323–328]. It now seems clear that the human C22 PUFA synthesis pathway consists of two succeeding elongation cycles (leading to 24:5(n-3)) followed by a Δ 6 desaturation, all of which occur in the endoplasmic reticulum. After transfer of the fatty acid to peroxisomes, there is a specific β -oxidation chain-shortening to the C22 product. By this route, the synthesis of DHA from acetyl-coenzyme A (acetyl-CoA) requires approximately 30 distinct enzyme activities and nearly 70 reactions, including the four repetitive steps of the fatty acid synthesis cycle [329].

Over the past few years, sequences encoding virtually all the enzyme activities involved in microsomal PUFA biosynthesis have been isolated, identified, and expressed in a variety of heterologous hosts (Table 4) [330]. Results from these studies help to increase our understanding of the biochemistry of elongases and desaturases and the regulation of PUFA biosynthesis. Then, the

Table 4 Origin of presently available genes for cDNAs encoding desaturases and elongases involved in the biosynthesis of LC-PUFA. The encoded enzymes have been characterized by functional expression. The numerous sequences published for the ubiquitous $\Delta 9$ -, $\Delta 12$ - and $\Delta 15$ -desaturases are not included [308]

Enzyme	Organism	Reference
$\Delta 4$ desaturase	<i>Thraustochytrium</i> sp	[322]
$\Delta 5$ desaturase	<i>Homo sapiens</i>	[331, 332]
	<i>Caenorhabditis elegans</i>	[333, 334]
	<i>Mortierella alpina</i>	[335, 336]
$\Delta 6$ desaturase	<i>Homo sapiens</i>	[337]
	<i>Caenorhabditis elegans</i>	[338]
	<i>Borago officinalis</i>	[339]
	<i>Ceratodon purpureus</i>	[340]
	<i>Physcomitrella patens</i>	[341]
	<i>Mortierella alpina</i>	[342, 343]
$\Delta 8$ desaturase	<i>Euglena gracilis</i>	[320]
(n-3) desaturase	<i>Caenorhabditis elegans</i>	[344]
$\Delta 6$ elongase	<i>Caenorhabditis elegans</i>	[314]
	<i>Mortierella alpina</i>	[317]
	<i>Physcomitrella patens</i>	[345]

next challenge will be the selection of a set of suitable copies of the available genes, which after transformation and expression in a heterologous host (such as oilseed crop) should result in a cooperating ensemble of enzymes and production of PUFA.

10.4.2

Anaerobic pathway

The diversity of PUFA synthesis described above relies on variations on desaturase and elongase biochemistry. This aerobic biosynthetic pathway was thought to be taxonomically conserved, but nature has also solved the problem of PUFA synthesis using a fundamentally different anaerobic pathway [329]. Indeed, numerous PUFA-producing bacterial strains are capable of producing PUFA under strictly anaerobic conditions, thus precluding the participation of an oxygen-dependent mechanism. The system involved here does not require the multiple desaturase and elongase enzymes outlined above, but instead uses a polyketide synthase-like (PKS) gene cluster, found in both prokaryotic and eukaryotic marine microbes, to synthesize PUFA [214].

Several marine bacteria contain EPA and DHA at levels as high as 25% of total membrane FA [346]. A genomic library prepared from one of these marine bacteria (*Shewanella* sp. Strain SCRC2738) was used to identify a 38 kb DNA fragment that resulted in EPA synthesis when expressed in *E. coli*. [347].

Experimental results [329, 348–350] indicated that these genes expressed in *E. coli* encode a protein complex that is capable of EPA synthesis without any reliance on enzymes of the *E. coli* FA or any long-chain intermediate such as 16:0-ACP. Apparently, the genes encode a PKS that acts independently of FA elongase and desaturase activities to synthesize EPA directly [213]. Genes with high homology to the *Shewanella* gene cluster have been identified in *Photobacterium profundum* [351] and in *Moritella marina*, which accumulates DHA rather than EPA [352]. Thus it is likely that the PKS pathway for PUFA synthesis is widespread in marine bacteria [213].

The thraustochytrid *Schizochytrium* sp accumulates large quantities of triacylglycerol rich in DHA (see chapter on thraustochytrids). Biochemical experiments have revealed the involvement of a PKS protein complex [329], which was further confirmed by molecular genetic analysis. The *Schizochytrium* genome encodes three proteins with domains highly similar to those encoded by genes from *Shewanella*, raising the possibility that the PUFA PKS has undergone lateral gene transfer [213].

Several “front-end” PUFA aerobic desaturases from *Thraustochytrium* have been identified recently [322, 353], but the prevalence of this newly discovered PKS-like biosynthetic pathway is not known. Because *Schizochytrium* is also a member of the Thraustochytriidae, it is perhaps surprising to find these two distinct biosynthetic pathways represented in the same family, but molecular characterization of PUFA biosynthesis in the Thraustochytriidae might provide insights into the evolution of this important pathway.

The primary structure of *Shewanella* PKS (and its relatives) does not conform to any of the previously described classes of PKS proteins. Instead, it suggests the assembly of several multifunctional proteins into a complex. PUFA PKS carry out some of the same reactions as FA and use the same small protein (or domain), acyl carrier protein (ACP), as a covalent attachment site for the growing carbon chain. However, in these enzyme systems, the complete cycle of reduction, dehydration, and reduction seen in FA is often abbreviated, so that a highly derivatized carbon chain is produced, typically containing many keto and hydroxy groups as well as carbon-carbon double bonds in the trans configuration (Fig. 21). The linear products of PKSs are often cyclized to form complex biochemicals that include antibiotics, aflatoxins, and many other secondary products [329]. Since the double bond on the PUFA molecules were formed by dehydration and isomerization of keto groups in cycles of polyketide-forming chain elongation, reaction to the C20 PUFA may not be the direct precursor of C22 PUFA [354].

The relative simplicity of this PKS-like system makes it attractive in terms of transgenic production of LC-PUFA. For example, introducing and regulating the three *Schizochytrium* PKS-like open reading frames in a transgenic plant is relatively simple compared with the more than five desaturase and elongase genes required for the aerobic pathway [306]. In addition, the identification of new (PUFA-specific) PKS activities such as double-bond isomer-

please confirm spelling correction of triacylglycerol to triacylglycerol

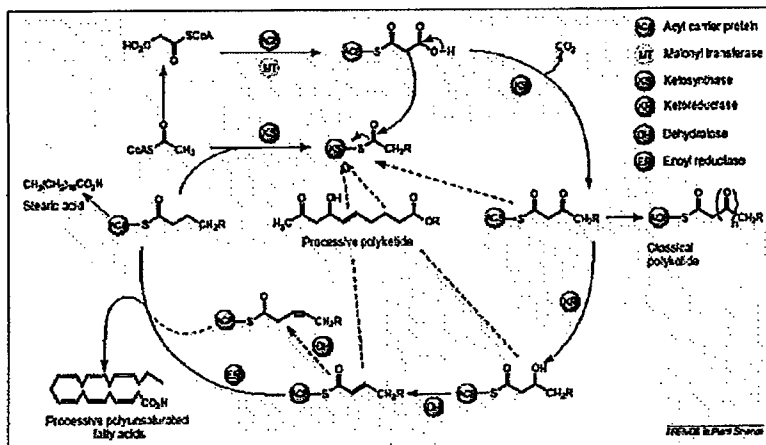


Fig.21 Generalized scheme for the processive synthesis of polyunsaturated FA (PUFA) by a polyketide synthase (PKS) system [214]. In the case of PUFA, it is envisaged that a primer molecule (in the form of acetyl-CoA) undergoes several rounds of sequential reactions (keto-synthase, keto-reductase, dehydratase and enoyl reductase), resulting in repeated synthesis and fatty acyl chain (esterified to the acyl carrier protein) elongation by two carbons per cycle. Because PUFA contain methylene-interrupted double bonds (i.e. at the third carbon), it is likely that a dehydratase (FabA-like) module in the PKS also simultaneously carries out a trans-cis isomerization to generate this configuration

ization might help in the bioengineering of additional families of polyketide antibiotics [214].

Furthermore, our improved knowledge of PUFA synthesis in *Shewanella*, *Schizochytrium* and their relatives has implications for understanding food-web dynamics in marine ecosystems. Because these organisms are significant primary producers of 20- and 22-carbon PUFA in cold-water oceans [346], the PKS pathway may be an important source of PUFA for fish and mammals and thus also for human diets. The importance of 20:5 in food-web dynamics of freshwater ecosystems has recently been discussed [355]. Finally, the identification of these PKS systems in ancient lineages raises intriguing questions about the evolutionary relationship of this newly discovered pathway to 20:5 and 22:6 FA relative to the enzymatically more complex desaturase/elongase route found in higher eukaryotes [213].

10.5

Some promising sources of marine LC-PUFA

10.5.1

PUFA from nonphotosynthetic microorganisms

If nowadays conventional fish oils are the main industrial sources of PUFA they may be not suitable to meet the increasing markets, notably for DHA owing to their limited supply (see section on sources and market), lower content of DHA in comparison to that of EPA, and peculiar taste and odour. Thus, the production of (n-3) PUFA by microbial fermentation of oleaginous microorganisms has attracted considerable attention in relation to industrial application of single-cell oil (SCO) [354, 356, 357]. For instance, a range of autotrophic and heterotrophic microbes has been assessed for potential commercial sources of EPA and DHA by various workers including Barclay et al. [358], Lewis et al. [359] and Vazhappilly and Chen [360], and the results of many studies in this area have been reviewed by Singh and Ward [361] and Rattedge [362].

In comparison to autotrophic microorganisms, the *de novo* synthesis of (n-3) and (n-6) PUFA by heterotrophic microorganisms may provide an easier and less expensive means of producing PUFA-rich biomass and oils [363]. Thus, Rattedge [362] considered that, despite improvements in the efficiency of photobioreactors, it is doubtful whether the growth of microalgae in bioreactors could be scaled up to satisfy even a modest demand for SCO rich in (n-3) PUFA, and suggested that heterotrophic microbes might be a more productive source.

In recent years, interest in the use of microheterotrophs as a source of PUFA has increased [364]. Microheterotrophs do not require some of the elements necessary for the culture of autotrophs (e.g., light, carbon dioxide), and some see them as a potential alternative to traditional commercial sources of PUFA. Arachidonic acid has been produced in quantity by some fungi [365, 366]. Certain bacteria have been shown to produce EPA and DHA [367, 368]. The recognized need in aquaculture for alternative sources of PUFA for feeding both larvae and adults has seen PUFA-producing bacteria successfully demonstrated as a means to enrich rotifers (*Brachionus plicatilis*, a live-fed organism for finfish larvae) with these FA [369–371]. In addition an increasing body of research into microheterotrophic PUFA production has concentrated on the thraustochytrids [363].

10.5.1.1

Bacteria

In marine food webs, microalgae have long been considered as the only *de novo* source of BPA [372]. Indeed PUFA were once thought to be absent in

bacterial membranes [373] and the production of PUFA by bacteria is often ignored [374]. However, numerous bacterial species of marine origin have now been shown to produce LC-PUFA such as EPA and DHA and some authors correctly pointed to the potential role of prokaryotic PUFA production in marine food webs [25, 347, 375].

Phylogeny

The screening of PUFA-producing prokaryote has led to the identification of previously undescribed taxa within the genera *Shewanella* and *Colwellia* [376, 377]. Subsequently, the chemotaxonomy of PUFA-producing bacteria was reviewed [30]. Indeed, until recently the taxonomic status of PUFA-producing bacteria received little attention, with most research efforts focused on the description of PUFA production [31].

Figure 22 represents the division-level diversity of the bacterial domain based on 16S rDNA sequences. Several of the divisions (e.g. Proteobacte-

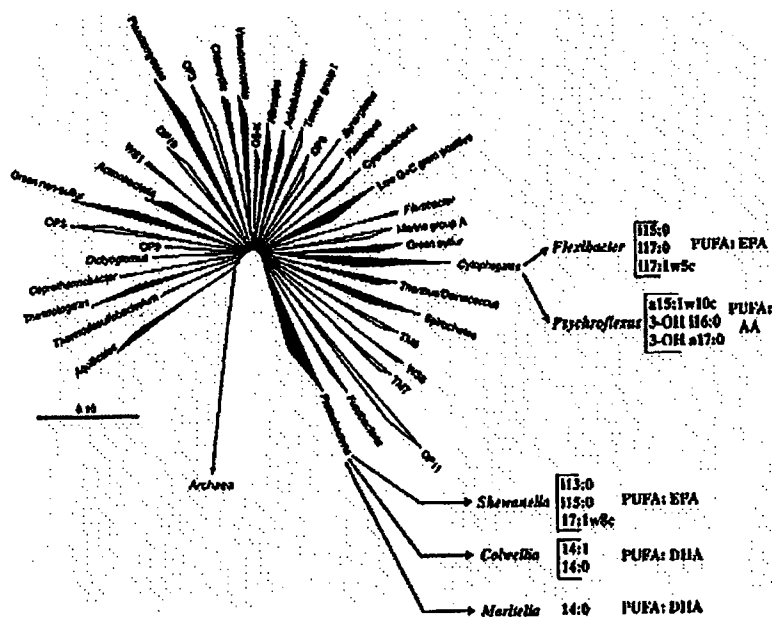


Fig. 22 Evolutionary distance tree of the bacterial domain [31]. The major PUFA-producing genera, *Shewanella*, *Colwellia* and *Moritella*, are expanded from the *Proteobacteria* division together with *Flexibacter* and *Psychroflexus* from the *Cytophagales* division. For each genus, biomarker FA and the types of PUFA are listed

ria, Actinobacteria) are well represented by cultivated strains. However the majority are poorly represented by cultured organisms. In fact 13 of the 36 divisions are defined by environmental sequences only [378]. The proportion of cultivated versus uncultivated sequences obtained from environmental molecular analyses may be used as an indication of the level of described biodiversity for a particular division. The occurrence of bacteria with the ability to produce PUFA is limited to five well-known marine genera which fall within two distinct domains of bacteria. Although separated by a significant evolutionary distance, the ability to produce EPA is apparent from both groupings. Species from each domain also possess the ability to produce further PUFA products, namely AA or DHA, respectively (Fig. 22). Thus, the ability to produce PUFA exhibits a phylogenetic linkage centred on two distinct lineages, the marine genera of the γ -Proteobacteria (*Shewanella*, *Colwellia*, *Moritella*, *Psychromonas*, *Photobacterium*) and more limited species within two genera of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) grouping (*Flexibacter*, *Psychroflexus*). However, it is pertinent to note that not all species within these genera express the ability to produce PUFA (Figs. 22, 23). Evidence implies that PUFA production is associated with physiological adaptations within marine bacteria.

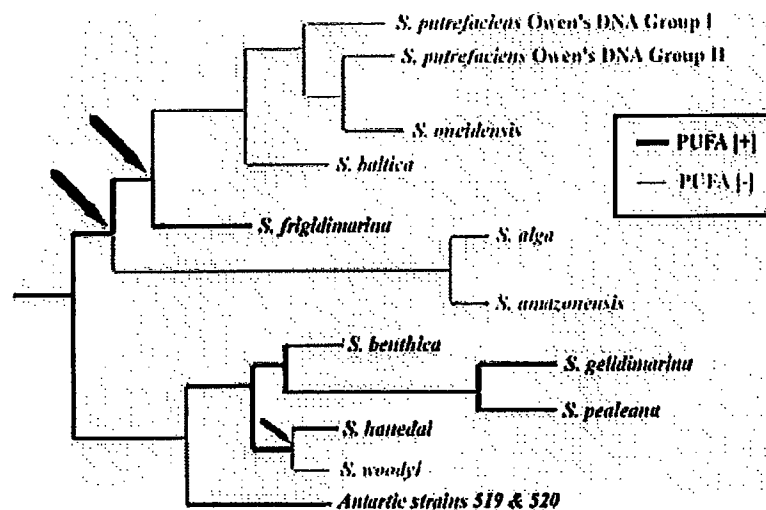


Fig. 23 Schematic of the phylogenetic relationship of the genus *Shewanella* based on 16S rDNA sequence. Species known to produce polyunsaturated FA (PUFA) and their lines of descent are highlighted in red. Species and lines of descent known not to produce PUFA are highlighted in blue. Arrows indicate sites of divergence where the expression of PUFA synthesis has been lost. Adapted from Russell and Nichols [30]

Marine ecology

Bacterial PUFA-producing isolates have been found to be particularly prevalent in high-pressure low-temperature deep-sea habitats and permanently cold marine environments [357, 367, 379]. Indeed, the majority of the γ -Proteobacteria PUFA producers are characterized as being psychrophilic³, halophilic and predominantly piezophilic⁴ or piezotolerant [30, 380]. These physiological traits have influenced the ecological distribution of PUFA-producing bacteria in the marine environment (Table 5). The enrichment of PUFA-producing strains from these environments has led to speculation that PUFA synthesis is an important adaptation for countering the effects of elevated hydrostatic pressure and low temperature on membrane fluidity or phase. In strains which have been analysed, PUFA synthesis undergoes temperature-dependent and, for deep-sea isolates, pressure-dependent regulation. Typically, as cultivation temperature is decreased, and/or pressure increased, PUFA incorporation into membrane phospholipids is enhanced. This modulation is thought to maintain appropriate membrane physical structure. Indeed, in marine bacteria, PUFA are component FA of certain phospholipids which occur in the cell membrane. It is considered that the low melting temperature of these highly unsaturated membrane components, combined with their unique molecular geometry in the membrane, yields a particular advantage at low temperature in balancing the competing homeoviscous and homeophasic forces in the cell membrane [30].

However, if bacteria of the CFB grouping are similarly psychrophilic and halophilic, they lack the ability to grow at high pressure [380]. In addition, for at least one high-pressure-adapted deep-sea bacterium, *Photobacterium profundum* strain SS9, growth at high pressure and low temperature does not depend upon PUFA synthesis [351]. Hence while PUFA production appears as a phylogenetically linked genotypic strategy for such selective pressures, their presence may not be essential for the growth of bacteria in such environments.

While there is not necessarily a phylogenetic relationship between psychrophilic organisms, such relationships may exist through the evolution of adaptive strategies for low-temperature growth. This is supported by empirical observations such as in the genus *Shewanella* (Fig. 23). Here, there is a good correlation between those species which are cold-adapted and produce PUFA (*S. pealeana*, *S. hanedai*, *S. benthica*, *S. gelidimarina*, *S. frigidimarina*), in contrast to those which do not produce PUFA and grow at higher temperatures (mesophiles) (*S. putrefaciens*, *S. alga*, *S. baltica*, *S. woodyi*, *S. oneidensis*, *S. amazonensis*). However, as indicated by Nichols

³ The term psychrophile (psychro = cold, phile = loving) is an operational definition, to describe the temperature-growth relationship of microorganisms [400].

⁴ (Piezo = pressure, phile = loving). Same comments as above.

Table 5 Major bacterial genera responsible for the production of PUFA in the marine environment (adapted from Nichols [25])

Species	Ps	Ha	Pi	PUFA	Environmental source	References
Shewanella						
<i>S. algae</i>	-	-	-	-	Red algae, Japan	[376]
<i>S. amazonensis</i>	-	+	-	-	Water, Amazon river	[381]
<i>S. baltica</i>	-	-	-	-	Oil brine, Japan	[376]
<i>S. benthica</i>	+	+	+	+	Holourithan intestine	[376]
<i>S. colwelliana</i>	-	+	-	-	Aquaculture, USA	[31]
<i>S. frigidimarina</i>	±	-	-	+	Sea ice, Antarctica	[376]
<i>S. Gelidimarina</i>	+	+	±	+	Sea ice, Antarctica	[376]
<i>S. hadenai</i>	+	+	±	+	Sediment, Artic	[376]
<i>S. japonica</i>	±	-	-	+	Sediments, mussels	[382]
<i>S. livingstonensis</i>	±	-	-	nd	Sea water, Antarctica	[383]
<i>S. oneidensis</i>	-	-	-	-	Lake sediment, USA	[31]
<i>S. pealeana</i>	±	+	-	+	Squid gland	[31]
<i>S. putrefaciens</i> OG1	-	-	-	-	Butter, UK	[31]
<i>S. putrefaciens</i> OG3	-	-	-	-	Butter, UK	[31]
<i>S. woodyi</i>	±	+	-	-	Sea water, Hawaii	[31]
<i>S. violacea</i>	+	+	+	+	Deep-sea	[384]
Colwellia						
<i>C. demingiae</i>	+	+	nd	+	Sea ice, Antarctica	[377]
<i>C. hadaliensis</i>	+	+	+	nd	Deep-sea	[385]
<i>C. hornerae</i>	+	+	nd	+	Sea ice, Antarctica	[377]
<i>C. maris</i>	+	+	nd	+	Sea water, Japan	[386]
<i>C. psychroerythraea</i>	+	+	nd	+	Flounder eggs	[377]
<i>C. psychrotropica</i>	+	+	nd	+	Burton lake, Antarctica	[377]
<i>C. rossensis</i>	+	+	Nd	+	Sea ice, Antarctica	[377]
Moritella						
<i>M. japonica</i>	+	+	±	+	Deep-sea	[384]
<i>M. marina</i>	+	+	±	+	Sea water	[387]
<i>M. vavanosii</i>	+	+	+	+	Deep-sea	[388]
<i>M. viscosus</i>	+	+	±	nd	Fish	[389]
Psychromonas						
<i>P. antarcticus</i>	+	+	+	nd	Sea-ice, Antarctica	[390]
<i>P. kaikoe</i>	+	+	+	+	Deep-sea	[391]
<i>P. marina</i>	+	+	+	+	Seawater	[392]
Psychroflexus						
<i>Ps. gondwanense</i>	-	-	nd	-	Burton lake, Antarctica	[393]
<i>Ps. torquis</i>	+	+	nd	+	Sea ice, Antarctica	[393]
Photobacterium						
<i>Ph. profundum</i>	+	+	+	+	Deep-sea	[394]

Ps: Psychrophilic, Ha: Halophilic, Pi: Piezophilic

et al. [31], *S. frigidimarina* and *S. pealana* are rather psychotolerant than psychrophilic and in addition *S. frigidimarina* also grows without the presence of salt. Thus these authors point out that the correlation among psychrophily, halophily and PUFA production is therefore not exclusive. They suggest that PUFA production may be a common physiological strategy for coping with the combined constraints of low temperature and marine salinity.

In addition to cold environment (sea ice) and deep sea water, PUFA-producing bacteria have also been isolated in the intestinal contents of marine fish and invertebrates [347, 368, 379, 395–397]. Moreover, the transfer of bacterially derived FA and specifically PUFA, between marine bacteria and higher trophic levels has been demonstrated [370, 398]. For example, recent studies have demonstrated that PUFA-producing bacteria can be used to enrich rotifers in EPA or DHA [370, 371, 399]. The greatest level of EPA enrichment in rotifers was 5.8% dry weight [370], whereas for DHA it was 0.3% dry weight [371]. These studies provide an important step in the development of novel sources of PUFA for aquaculture [346].

All these facts uphold the idea presented by Nichols [25] that the assumption that microalgae provide the bulk of de novo PUFA production for all marine food webs must now be actively reviewed to determine the role and potential importance of PUFA-producing prokaryotes in marine microbial niches such as sea ice, marine animals and abyssal communities.

Biotechnology

Interest in the production of PUFA from alternative sources for use in aquaculture feeds and human nutraceuticals (see section on health benefit) has fuelled recent research into the molecular biology of PUFA production in prokaryotes. A key advantage of bacterial PUFA production (as for thraustochytrid PUFA production, see below) is that only a single PUFA is produced, rather than the complex mixture yielded from fish or algal oils [30]. Thus bacterial sources of PUFA remove the expense of preparative purification in the production of high-purity PUFA oils.

In addition to their potential use as "cell factories", bacteria in particular offer the biotechnological opportunity to investigate the genes and enzymes responsible for PUFA production. A variety of bacterial fatty-acid biosynthetic mechanisms exist, which vary with taxonomic identity and class of fatty acid product [401–403]. Some reports have suggested that bacterial (n-3) PUFA production is mediated by undefined desaturases [30, 348, 352]. However, as indicated by Allen et al. [351], sequence studies of bacterial genes required for PUFA biosynthesis have gradually led to a reappraisal of this view (see section on biosynthesis).

Initial insight into the genetics of bacterial PUFA synthesis was gained by the transfer of a gene cluster from *Shewanella putrefaciens* SCRC-2738 into *Escherichia coli* and a *Synechococcus* sp. resulted in the successful expression

of EPA in these organisms [347]. However, the level of expression achieved was low. The gene cluster used in both cases consisted of a 38 kb fragment containing eight open reading frames with three of these possessing homology with genes that encode for enzymes involved in fatty-acid biosynthesis. Further characterization using these organisms has identified five genes responsible for PUFA biosynthesis, designated ORFs 2, 5, 6, 7 and 8. A subsequent analysis of the predicted amino acid sequences of the products of these genes indicated that they are most related to microbial polyketide synthase (PKS) complexes and fatty acid synthase (FA) enzymes (see section on biosynthesis). In addition to the *Shewanella* sp. SCRC-2738 sequences, related genes partially responsible for PUFA production have been analysed from the DHA-producing bacterium *Moritella marina* strain MP-1 (formerly *Vibrio marinus*) [352] and from a DHA-producing thraustochytrid marine protist belonging to the genus *Schizochytrium* [329]. Recently, Metz et al. [329] reported biochemical analyses of PUFA production in *E. coli* strains harbouring *Shewanella* sp. SCRC-2738 DNA and in the *Schizochytrium* species. Consistent with the examination of enzyme domains, isotopic-labelling studies provided compelling support for a PKS-like pathway of PUFA synthesis in both systems studied [329].

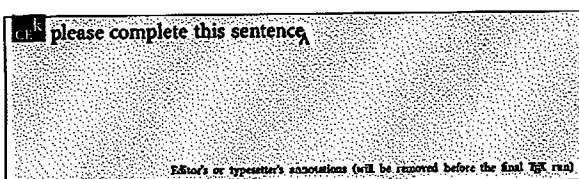
After spending significant effort on understanding the mechanistic microbial production of PUFA, research now has to focus on the regulation of such biosynthesis to maximize the transgenic potential of bacterial LC-PUFA genes.

10.5.1.2

Thraustochytrids

Thraustochytrids are microheterotrophs generally considered necessarily marine with a specific requirement for Na^+ ions [404] that feed as saprobes or occasionally as parasites [405]. Thraustochytrids have been characterized by the presence of sagenogenetosome, an ectoplasmic net, a cell wall with non-cellulosic scales, and a life cycle consisting of vegetative cells, zoosporangium, and biflagellate zoospores [354]. They have a wide geographic distribution in estuarine and marine habitats, with strains isolated from Antarctica [406], the North Sea [407], India [408], Micronesia [409], Japan [410], Australia [359] and Fiji [354]. They have been reported frequently from seawater, sediments, algae, and invertebrates, both as saprotrophs and parasites. Their ubiquitousness and physiological capabilities to utilize a wide variety of substrates suggest that they play a definite role in the marine ecosystem.

Originally thought to be primitive fungi, thraustochytrids have more recently been assigned to the subclass Thraustochytridae (Chromista, Heterokonta), aligning them more closely with the heterokont algae (e.g., brown algae and diatoms) [411].



In research on microheterotrophic PUFA production, particular attention has been given to the thraustochytrids since their lipids contains proportionately large quantities of ω 3 PUFA and particularly DHA [357].

Bowles et al. [386] have screened 57 thraustochytrids isolates from three different locations. Although a common fatty-acid profile for the thraustochytrid isolates emerged ((n-3) PUFA as a significant component, as previously found [?]), there was considerable difference in the DHA content of the oil. This large variation in DHA proportion can also be extended to biomass and lipid yields depending on the thraustochytrid strains (Table 6). Thus, in some isolates from a cold-temperate environment, DHA can represent almost 50% of the total FA present while those from a sub-tropical environment produce higher levels of biomass, with up to 37 (w/w) % oil but with a lower DHA content [423].

As indicated by Lewis et al. [363], most reports concerning the production of PUFA by thraustochytrids have dealt almost exclusively with DHA production (Table 6), as this compound is often the most abundant PUFA produced by strains of thraustochytrids reported to date. However, it is evident that some thraustochytrid strains also produce other PUFA. Thus, Huang et al. [354] have shown that the fatty acid profiles of DHA-producing thraustochytrids could be used to classify them into five separate categories:

- DHA/DPA (docosapentaenoic acid; C22:5 (n-6)),
- DHA/DPA/EPA,
- DHA/EPA,
- DHA/DPA/EPA/AA
- DHA/DPA/EPA/AA/DTA (docosatetraenoic acid, C22:4 (n-6)).

Their seven isolates from Japan and Fiji were proved to be new thraustochytrids by their specific insertion sequences in the 18S rRNA genes. The phylogenetic tree constructed by molecular analysis of 18S rRNA genes from those isolates and typical thraustochytrids shows that strains with the same PUFA profile form each monophyletic cluster. These results suggest that the C20-22 PUFA profile may be applicable as an effective characteristic for grouping thraustochytrids. Moreover, Bowles et al. [423] have shown that, among their 57 thraustochytrids, all synthesized the ω 6 PUFA arachidonic acid in varying amounts, mainly as a minor component of the PUFA and that EPA was present in the oil produced by all the isolates except two (however EPA content was generally low, varying from 0.2 to [-(%w/w)]0.6 of the dried thraustochytrid cells). So, although about 15 strains of thraustochytrids, including *Thraustochytrium aureum*, *T. roseum*, *T. aggregatum*, *Schizochytrium limacinum* and *S. aggregatum*, have been reported to produce significant amounts of DHA (Table 6), there are many potential strains yet to be explored [363, 424].

As indicated by Hammond et al. [425], there are no reports in the literature of direct human consumption of thraustochytrids. This is due to the fact that, prior to the late 1980s, thraustochytrids had never been cultured

Table 6 Docosahexaenoic acid (DHA) production by thraustochytrids [350]

	Age (d)	Temp (°C)	Vessel	Other	Biomass (g/L)	Lipid (% dw)	(% TFA)	(mg/g)	(mg/L)	Refs.
Shizochytrium sp. SR21	2.5	28	Fermenter	PH. 4	21	50	35	224	4700	[412]
Shizochytrium sp. SR21	4		Fermenter 300 rpm	–	48	77	36	277	13 300	[413]
S. Limacium SR21	5	25	Flask	–	38	37	33	110	4200	[357]
S. aggregatum ATCC 28209	10	25	Flask 200 rpm	Dark	0.9	–	1.7	30	0.4	[360]
Thraustochytrium aureum ATCC 34304	6	25	Flask 300 rpm	Light	3.8	16.5	49	70	270	[414]

Table 6 (continued)

	Age (d)	Temp (°C)	Vessel	Other	Biomass (g/L)	Lipid (% dw)	(% TEA)	(mg/g)	(mg/L)	Refs.
<i>T. aureum</i> ATCC 34304	6	25	Flask 300 rpm	Light	4.9 20.3 51 104 511 1					[415]
<i>T. aureum</i> ATCC 34304	2.5	25	Flask	Light	5.7	8.1	40	—	—	[356]
<i>T. aureum</i> ATCC 28211	6	25	Flask 200 rpm	Dark	0.8	—	3.7	50	4.0	[360]
<i>T. roseum</i> ATCC 28210	5	25	Flask 250 rpm	Light	7.6	18.2	50	87	650	[416]
<i>T. roseum</i> ATCC 28210	12	25	Flask 250 rpm	Fed batch	17.1	25	49	115	2100	[417]
<i>Thraustochytrium</i> sp. ATCC 20892	4	25	Flask 200 rpm	—	2.7	7.3	35	25	68	[418]
<i>Thraustochytrium</i> sp. ATCC 20892	6	28	Flask 120 rpm	Light	7.5	32	25	—+	—	[419]

on a scale larger than a laboratory shake flask. Barclay [422] and Bajpai et al. [414, 415] were the first to successfully cultivate *Schizochytrium* sp. and *Thraustochytrium* sp., respectively, in fermenters (two patents have been filed detailing the cultivation of thraustochytrid strains to produce lipids containing EPA and DHA [422, 426]). However, thraustochytrids are primarily consumed by filter-feeding invertebrates in the marine ecosystem, including mussels and clams, and by fish that are consumed directly by humans. Thus, in the last few years, thraustochytrids have been successfully used for commercial production of PUFA-rich products notably in aquaculture applications. This is the case for a *Schizochytrium* strain, which is the basis for two products marketed for enriching rotifers (*Brachionus* sp.) and brine shrimp (*Artemia* sp.) with PUFA, prior to feeding these organisms to cultured finfish larvae ([427]; www.aquafauna.com; www.sandersbshrimp.com). OmegaTech commercialized a product for aquaculture applications (HUFA 2000, a spray-dried form of *Schizochytrium* sp. dried microalgae), which has been successfully utilized for over seven years as an excellent stable dietary source of DHA in shrimp larvaculture and finfish (red seabream, Japanese flounder) culture with no adverse effects. Use of *Schizochytrium* sp. in these applications has been found to promote larvae survival and growth [428]. Other uses of thraustochytrid oil are being actively explored. Monsanto (www.monsanto.com) is producing *Schizochytrium* sp.-derived oil under a cooperative technology agreement with OmegaTech (www.omegadha.com). Moreover, dried *Schizochytrium* microalgae (DRM, OmegaTech) have also been generally recognized as safe (GRAS) for use as a DHA-rich ingredient in broiler chicken and laying-hen feed at levels up to 2.8–4.3%, respectively [429]. Since 1997, DHA-enriched eggs from hens fed a diet containing approximately 1% DRM are now commercially marketed in the United States, Mexico, Germany, Spain, Portugal, the Benelux countries, Italy, Norway, and Israel [430].

These products have entered the market in direct competition with microalgal and fish-oil products. It is possible, however, that thraustochytrids will offer some advantage over other oils as sources of PUFA for aquaculture. Many aquaculture species require proportionally more DHA than EPA in their diet [431]. The PUFA profiles of many thraustochytrids fit this criterion, while most oils from the fish-meal industry contain more EPA than DHA. However it has to be noticed that a recent study [432] has revealed that the replacement of fish oil with a dried product made from a thraustochytrid culture in canola-oil-based diets for Atlantic salmon could affect the disease resistance of fishes. Indeed, if the authors didn't observe significance differences in final weight, weight gain, feed consumption, feed efficient ration or protein value between the diets, nor in whole-body chemical composition, organ somatic indices or measures of immune function, they have noticed that, following transfer to seawater and two challenges with *Vibrio anguillarum*, cumulative mortality was significantly lower in fish fed some

fish oils than the others. They concluded that their thraustochytrid strain had no detrimental effects on the performance of salmon although, at the current inclusion of 10%, it failed to confer the same effects as fish oil under challenging conditions.

In conclusion, as indicated by Lewis et al. [363], thraustochytrids are clearly a new and potentially competitive player in the PUFA market. Considerable work is required before the production of oil from these organisms significantly increases its share of the market for PUFA-rich products. To achieve this aim, the following key stages need to be negotiated. Firstly, the collection, screening, and maintenance of PUFA-producing strains. Several strains with potential for the commercial production of DHA-rich oils have been isolated already. However, if thraustochytrids that produce higher yields, more attractive PUFA profiles, or other less common but sought-after PUFA are isolated and optimized, then demand for these isolates and compounds may well increase. Secondly, the efficiency of PUFA production must be optimized. The types and amounts of PUFA produced by individual strains of thraustochytrids are susceptible to manipulation by varying culture conditions. Enhancement of PUFA profiles using molecular techniques may be also considered. Different markets will provide demand for strains that produce high levels of PUFA measured either in terms of biomass (i.e., PUFA production w/w cell mass) or volume (i.e., PUFA production w/vol fermentation medium). Thirdly, appropriate conditions for long-term storage of microbial cells and their products must be determined. The form and stability of thraustochytrid biomass and of oils will be major factors in determining the suitability of these products for use as food additives. Finally, oil extraction and refinement technologies must be developed to meet market demands for cost-effective and safe trophic transfer of PUFA to the target consumers. The bottom line for the biotechnological future of thraustochytrid oils will be their competitiveness against other PUFA-rich oils. Nevertheless, oils derived from fish and microalgae generally have a complex fatty acid (total and polyunsaturated) profile, and do not readily lend themselves to the isolation of high-purity (> 98%) FA. Conversely, oils produced by some thraustochytrids have relatively simple fatty-acid profiles and may well be more amenable to cost-effective refinement. DHA is a good illustration of the industrial potential of thraustochytrids. Thus, the largest potential market for microbial oils containing DHA is perceived to be as an additive to infant formulae as an essential fatty acid for brain and retinal development (see section on health benefit); Ratledge [362] considered that the presence of significant quantities of EPA in the thraustochytrid oils so far assessed precluded its use for this purpose. Indeed, eicosapentaenoic acid is considered contraindicated in breast milk substitutes, but strain selection will easily allow this difficulty to be overcome [423].

10.5.1.3

Conclusions

Growing interest in PUFA applications in various fields coupled with their significance in health and dietary requirements (see section on health benefit) has encouraged "hunting" for more suitable sources of these compounds. The inadequacy of conventional agricultural and animal oils has put attention on developing new microbial technologies. Indeed, microorganisms represent the largest reservoir of undescribed biodiversity, and hence possess the greatest potential for the discovery of new natural products. It is estimated that the Earth currently supports 3–30 million species of organisms. Of these, approximately 1.4 million have been described by science. This includes virtually all the species of birds and mammals (~13 500). In contrast only around 200 000 of the estimated 1.0–1.5 million species of fungi have been characterized (i.e. 13–20%). For the bacteria this percentage is even lower with estimates ranging from only 1–10% of probable species being described in culture [433, 434].

However, the focus of biotechnology on highly valuable PUFA requires knowledge of how microorganisms control and regulate the fatty-acid biosynthetic machinery in order to obtain specific PUFA in high yield.

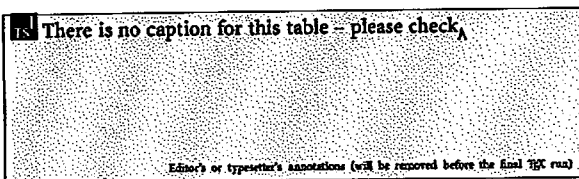
Elucidation of the signalling systems and mechanisms transmitting the signals from different membranes to the major sites of lipid biosynthetic machinery represents a challenging and potentially rewarding subject for further research [435]. At least, the extensive research and development of PUFA production carried out over the past few years will be aimed at improving the economic competitiveness of microbial lipids compared to plant- and animal-derived oils.

Nowadays, by using conventional stirred-tank fermenters, economically viable quantities of certain microorganisms that are rich in LC-PUFA can be produced [436]. The chief advantages of such techniques lie in the consistency and purity of the final fatty acid product. Further, unlike the scenario with fish oils, economies of scale have reduced the price of oils derived from organisms raised in fermenters by 10- to 30-fold [437].

10.5.2

PUFA from fish

Fish is a major source of food for mankind, providing a significant amount of the animal protein diet in many countries. Moreover, the consumption of fish has been linked to health benefits (see section on health benefit). Indeed, oils from fish are characterized by a large range of FA from 12–26 carbon atoms and 0–6 double bonds. The bulk of the fatty acid chains is contributed by saturated (15–25%), monoenes (35–60%) and polyenes (25–40%). In contrast with the other fats and oils, fish oils contain large amounts of EPA and



DHA, respectively, 14–19% and 5–8%. The proportion of polyunsaturated FA depends on many parameters (see below). Saturated FA include C12 up to C24:0 components, and some branched chains (iso C16, iso C17) are also found. Among the monoenes, 16:1(n-7), 20:1(n-9) and 22:1(n-11) are present in various amounts, this last component being bioconverted from the corresponding fatty alcohol of copepod wax ester by the fish liver [438]. More than 50 different FA were described in marine fish oil, but eight species frequently represent more than 80% of the total amount (Table 7).

In fish tissues, the composition of FA (mainly of triacylglycerols and to a lesser extent of phospholipids), is determined by diet composition and lipid metabolism [439, 440]. Fish have the ability to synthesize *de novo* the saturated and monounsaturated FA and also to selectively absorb and metabolize dietary FA including LC-PUFA [440, 441] in order to obtain an optimal fatty acid composition [442]. This optimal composition seems to be a characteristic for each species and even each strain [443, 444]. Moreover, the PUFA conversion capacity in fish varies among species and even races [439]. Thus, freshwater fish are generally able to elongate and desaturate α -linolenic acid (18:3(n-3)) to EPA and DHA, whereas marine fish, which lack or have a very low activity of Δ 5-desaturase, cannot and require LC-PUFA such as EPA and DHA in the diet [440].

In addition to food accessibility and lipid metabolism some environmental parameters also notably influence the proportion of PUFA [445]. Indeed, the colder the water, the higher the amount of these components. An inherent property of cells in poikilothermic animals is their capacity to adjust the physicochemical characteristics of their membranes to the prevailing temperatures. This phenomenon, known as homeoviscous adaptation of membrane fluidity, has been reported in poikilothermic fish [446]. In fish, during adaptation to reduced temperatures, unsaturation of the constituent FA increases, with the polar head group as well as the molecular species composi-

Table 7

	Menhaden	Herring
14:0	7–12	5–8
16:0	15–26	10–19
16:1(n-7)	9–16	6–12
18:0	2–4	1–2
18:1(n-9)	8–14	9–25
20:1(n-9)	–	7–20
20:5(n-3)	11–16	4–15
22:1(n-11)	< 1	7–30
22:6(n-3)	5–14	2–8

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tion of membrane phospholipids being reorganized [202]. Evidence suggests that the fatty acid distribution is very individual from species to species and depends on many factors like season, temperature, fishing ground, fish species, age, gender or nutritional habits [447–452].

The FA distribution in triacylglycerols is stereospecific [www.cyberlipid.org]. Indeed, results indicate that the stereospecific location of the PUFA is in position 2 (also 3 for EPA in cod) in fish but in position 3 for mammals (same in seal, whale and polar bear). This raises the question of the availability of these FA from food expecting a benefic effect on diverse human functions (see section on health benefit).

Familiar fish species used in the production of fish oil include among others, anchovies, capelin, Atlantic cod, Atlantic herring, Atlantic mackerel, Atlantic menhaden, salmonids, sardines (see section on market and sources). Of the world's fish oil production, 90% is produced from fatty fish where lipids are localized mainly under the skin, around the intestines or in the white muscle. In such fishes, the oil content varies (see above) but it can reach 21% (herring) and 18% (sardines). Such oils are still the least-expensive natural source of preformed long-chain PUFA, and several industries (e.g., Ocean Nutrition, Halifax, N.S., Canada, and Pronova Biocare, Sandefjord, Norway) now specialize in their production and purification through cold pressing, further concentration by winterization (i.e., chilling), and other technologies [431].

There are, however, potential problems associated with fish oils as a source of PUFA such as: taste, odor, stability problems as well as the presence of co-extracted contaminants. Some could be at least partially solved for example by microencapsulation [453] and deodorization [454]. Nevertheless, the crucial problem of those oils is their sustainability due to the worldwide decline of fish stocks (see section on market). A better use of raw material but as well of by-catch and by-products from fisheries may be one solution, another is to look for other sources (see above) including non- or little-exploited fish species.

Lipid content has been studied for a long time mainly in the liver and edible parts of fish such as muscles. Several investigations have evidenced that other parts i.e. liver and skin, often discarded for several reasons when fish are prepared for consumption, may have possible nutritional and therapeutic value mainly due to their EPA and DHA content [445, 455]. Indeed valuable fish oils can be obtained from trash fish and/or fish scraps or cannery wastes, which are often called "gurry" from filleting and canning operations. Fish scraps normally consist of the head, skeleton, and adhering proteinaceous tissues.

Surprisingly, it can also be observed that often no detailed chemical studies of the lipid and fatty acid content of certain fish are available, even though the fish most often consumed or commercially exploited, such as for example the species *Sardinella* and *Cephalopholis* in Senegal waters [455].

Capelin, squid liver, krill (*Euphausia superba*) could constitute sustainable new sources [437].

10.6

Current utilization of marine oils and lipids

10.6.1

Market

10.6.1.1

Production

From a world production of oil and fat of about 20 million tons in 1939, about 77 million tons were produced in 1989 where 74% were of vegetal origin (soybean 19% > palm 13% > rapeseed 10.3% > sunflower 9.6%). In 2003–2004 the global production of fats and oils is expected to be 128.5 million tons with 82% of vegetal origin. The world average consumption of oils and fats in 2003 is about 20 kg per capita [www.cyberlipid.org].

Of the estimated 89 million tonnes of fish produced in 2000 in the world, excluding China, nearly 71 percent (63 million tonnes) was used for direct human consumption. The remainder (about 29 percent) was utilized for various nonfood products, mostly for reduction to meal and oil (the state of world fisheries and aquaculture, 2002 FAO). Indeed, nowadays, a third of the world's catch from the seas is going into manufacturing fish meal and fish oil. Thus, the world production of marine oils represent approximately 1% of the commodity world fats and oils production (Table 8).

Remark: the International Fishmeal and Fish Oil Organisation (IFFO) is the international nongovernmental trade organization representing fish meal and oil producers worldwide. It has more than 200 member companies in 38

Table 8 Production (million tonnes) for 17 commodity oils in the four-year period 1998/99 to 2001/02 [456]

	98/99	99/00	00/01	01/02
Total production	107.6	113.5	117.3	119.7
Soybean	24.60	25.30	27.10	29.40
Palm	19.40	21.30	23.70	24.30
Rapeseed	12.70	14.50	13.90	13.40
Sunflower	9.30	9.50	8.70	7.50
Other vegetal oils	19.70	20.50	21.70	22.40
Fish	0.86	1.38	1.42	1.12
Other animal fats and oils	21.04	21.02	20.78	21.58

countries. Two-thirds of the world's production of fish meal and fish oil are members of the IFFO and 95% of the exports of fish meal and oil are also part of the IFFO.

Chile, Peru, Scandinavia, USA and Japan are the main suppliers of fish oil (Fig. 24). The average world production between 1991 and 2001 was about

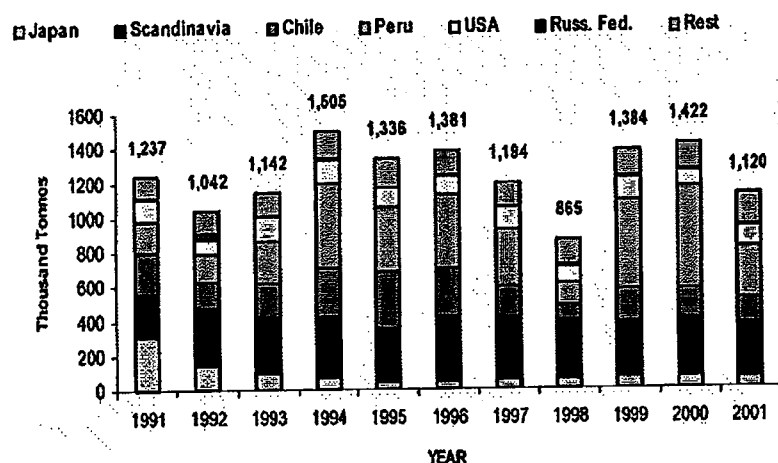


Fig. 24 World fish body oil production - major producers (IFFO)

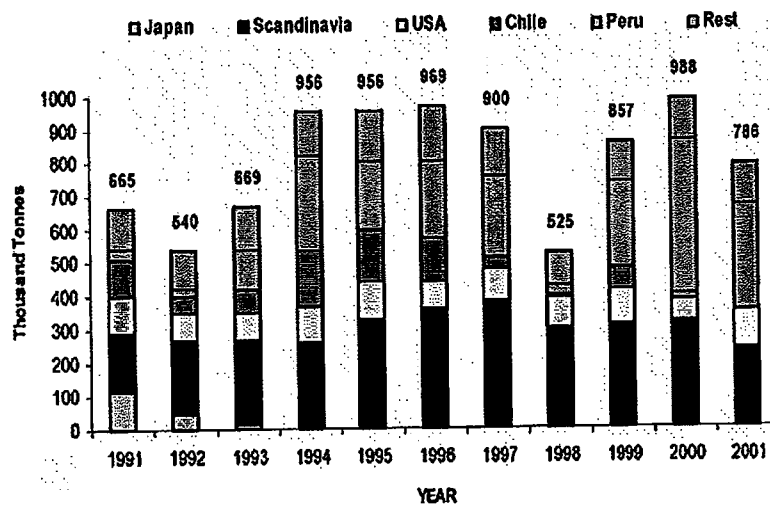


Fig. 25 World marine oils and fats exports by major exporters (IFFO)

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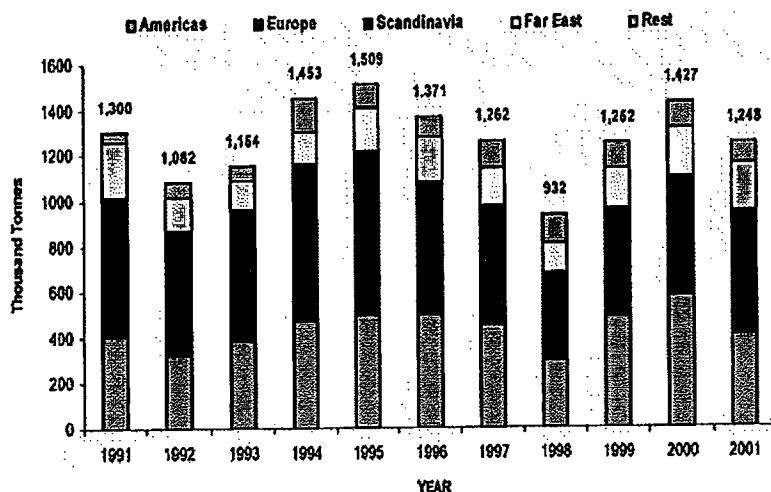


Fig. 26 World marine oil consumption and stocks – major consumers (IFFO)

1.25 million tonnes of fish oil produced annually. Important fluctuations in production can be observed, they were due to the El Niño phenomenon mostly in Chile and Peru. In 1998, which was the big El Niño year, the production of these two countries only reached 210 000 tonnes while the average over the last five years was 520 000 tonnes (the total catch volume by Peruvian fisheries was reduced to 3696 million tons, only 45% of the 2002 catch of 8238 million tons). However, due to better prediction of such climatic occurrence, the governments concerned are increasingly proactive in anticipating and taking precautionary measures for fishing. Thus, precautionary approach to fisheries management has been maintained (in Peru notably) to safeguard the viability and prevent depletion of stocks through overfishing. Careful management of the fishery and a return to normal environmental conditions allowed stocks to recover in 1999 and 2000.

10.6.1.2

Exportation

The major exporters are mainly the same countries, with the noticeable exception of Japan that is rather now a net importer (Fig. 25). Over the past decade fish oil exports by Peru (the main exporting country) have expanded by almost twelve times (US\$ 91.1 million in 2001). However, Peru's exportation is variable; it can be enormous (up to 500 000 tonnes in 2000) or very small, notably during El Niño periods. The second main fish oil exporter is now the USA (US\$ 41.7 million in 2001).

10.6.1.3

Consumption

Consumption of fish oils by countries is indicated in Fig. 26. Most of the fish oil goes into salmonid feed in Norway, Chile, Canada and various European countries, which is the reason for the predominance of these countries in terms of consumption.

Remark: fish oil is included in aquaculture feeds as a source of both dietary energy and PUFA. Considerable research is occurring worldwide in an effort to find alternatives to fishmeal and fish oil in aquaculture feeds. However, this research is tempered by the obligate dietary requirement of many marine finfish species for long-chain PUFA (LC-PUFA: e.g., EPA and DHA).

Aquaculture has been the world's fastest-growing food production over a decade [438]. The world aquaculture production has at least multiplied by a factor of two in the last ten years: 24 457 421 tonnes live weight in 1993 and 48 413 636 tonnes live weight in 2001 (Eurostat and FAO sources). The International Fishmeal and Oil Manufacturers Association [www.iffco.com] estimates that inclusion of fish oil in aquaculture feeds will rise from 380 000 tonnes in 1994 to 582 000 tonnes in 2001 and 1 133 000 tonnes in 2010 (Table 9). With aquafeed demand at about 1 million tonnes of fish oil in 2010, depending on the production of fish oil it could be around 80% or even close to 100%. This could well result in a worldwide undersupply of fish oil, leading to increased demand for fish oil alternatives. Moreover, this lack of fish oil will have an impact on aquafeed composition. It seems likely that cheap, plant- or animal-derived oils, which often contain low levels of LC-PUFA, will be used increasingly as alternative sources of energy in some aquaculture feeds. If such substitution does occur, sufficient LC-PUFA to meet the dietary requirements of cultured aquaculture species may be required from other sources. Typically, many cultured marine species require around 1% to 2 wt/wt % LC-PUFA in their diets [457, 458]. Pike and Barlow [459] estimated that marine aquaculture finfish species will require about 2×10^6 tonnes of feed in 2010.

Table 9 Fish oil use prediction based on an annual world production of 1.25 million tonnes for the period 2002–2010 [461]

	1990	2002	2010
Edible	76%	30%	14%
Industrial	8%	12%	5%
Aquafeed	16%	56%	79%
Pharmaceutical	–	2%	2%

Table 10 Predicted use of fish oil in fish feed (data from IFFO)

	% of fish oil inclusion in feed produced		Thousand tons of fish oil	
	2000	2010	2000	2010
Carp	–	0.5	–	103
Catfish	1.0	–	5	–
Tilapia	1.0	0.5	8	9
Milkfish	2.0	2.0	6	11
Shrimp	2.0	3.0	30	73
Eel	5.0	8.0	17	23
Marine fish ¹	10.0	12.0	23	156
Trout	15.0	15.0	95	121
Marine fish ²	20.0	15.0	226	335
Salmon	25.0	20.0	307	379
TOTAL			716	1209

¹ Flat fish including flounder, turbot, halibut, sole and cod, hake

² Bass, bream, yellowtail, grouper, jacks, mullets

These figures point to a potential demand, for these species alone, for at least 10 000 tonnes of LC-PUFA per annum (Table 10).

In addition to aquafeed, the current and potential world market for fish oil products spans a number of sectors from unprocessed, oil-rich biomass for animal feeds, to high-quality food-grade oils for use as food additives and nutraceuticals, and to very-high-purity oils and even individual FA for use in the pharmaceutical industry (Table 9).

As indicated by Lewis et al. [363], the imprecise boundaries surrounding the nutraceutical market make estimating the size of this market sector more difficult. Sales of marine supplement oils were in the order of \$55 million in the United States in 1996 [460], and represented 20% of sales from health food retail outlets. In the United Kingdom, fish oils account for approximately 29% (U.S. \$140 million) of the total annual market for nutraceuticals [461]. Moreover, there is an increasing trend for infant formula manufacturers to include PUFA-rich oils in their products. Typical inclusion levels of PUFA-rich oils are designed to achieve a final DHA concentration in dry infant formula of 0.1% to 0.2 wt/wt %. Indeed, the Western European market for infant formula increased from 81 500 tonnes in 1988 to 103 933 tonnes in 1994. Extrapolating these figures suggests a potential annual demand in the European infant formula market for up to 100 to 200 tonnes of DHA. Several food and beverage products enriched with DHA or other PUFA are already on the market. Mukherjee [461] reported the availability of products such as enriched spreads, breads, eggs, and soft drinks in Europe and Japan. Bread

enriched with refined tuna oil as a source of LC-PUFA is achieving substantial market penetration in Australia. As awareness by both consumers and regulators of the importance of adequate levels of PUFA in our diet increases, it can be assumed that demand for a greater range of PUFA-enriched products will increase [363].

10.6.1.4

Prices

The biggest use of fish oils is by the aquaculture industry, where it is necessary to have an oil rich in the long-chain polyunsaturated FA characteristic of fish oils. For this purpose, therefore, fish oil cannot be adequately replaced by vegetable oils. To meet this demand there has been a reduction in stocks and an increase in price. In 2000 and 2001 the average monthly price for crude fish oil ranged from US\$ 235–325/tonne and \$ 323–598/tonne. In January 2002 it was \$613/tonne. [Oil World, www.oilworld.org]. In August 2002, crude fish oil prices peaked (about 650 US\$ per ton) and have started to decline ever since. In November 2002, finally soybean oil prices managed to overtake those of crude fish oil due to the shorter supply than initially forecast, which make the latter competitive once more on the hardening market. For 2002, the average price of crude fish oil from any origin was about US \$587/tonne; in 2003 it was about US\$ 562/tonne (data from Oilworld). Peru's enormous fish oil production capacity sets this product's international prices.

10.6.2

Common resources

Fish oil is a by-product of industrial fishing and the fish meal industry. Fish oils are produced almost exclusively from small, bony species of pelagic fish (living in the surface waters or middle depths of the sea), for which there is little or no demand for human consumption [Fishmeal Information Network, www.fin.org.uk]:

South America (three species)

In Peru, anchovy is by far the most important species for fishmeal and fish oil production, with sardine largely making up the difference. The Chilean fishmeal industry uses anchovy, sardine and jack mackerel.

Europe (seven species)

Seven key species are used to produce fishmeal and fish oil in Europe. These can be divided into three groups:

- a) No use for human consumption (inedible feed-grade fish – sandeel, capelin, Norway pout).
- b) Potential use for human consumption but mainly used for fishmeal because of limited outlets for human consumption (blue whiting, sprat).

- c) Primary use is human consumption but surplus may be used for fishmeal (herring, horse mackerel).

Fishmeal production also provides a major outlet to recycle trimmings from the food-fish processing sector which would otherwise be dumped at extra cost to the environment and the consumer. In the EU, Spain, France, Germany, Ireland and the UK produce fishmeal and fish oil primarily from trimmings.

Global capture fisheries, i.e. catches of wild fish as distinct from farmed fish, are valuable and finite resources which, although renewable, are highly vulnerable. Moreover, overfishing has caused the collapse or near collapse of some valuable fisheries. Overexploiting one fish species can affect other species, not least birds and mammals, in the marine ecosystem. This situation has generated understandable and justifiable pressure for environmentalists to reduce fishing effort and catches further by introducing tighter regulatory measures [438].

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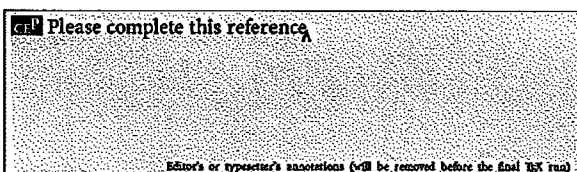
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The effect of various intakes of ω 3 fatty acids on the blood lipid composition in healthy human subjects¹

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ABSTRACT In a study with 52 healthy volunteers, the effect of different amounts of ω 3 fatty acids on the blood lipid composition was investigated. Doses of 1.4, 2.3, 4.1, and 8.2 g of ω 3 fatty acids were administered to these subjects daily over a period of 4 wk. Total cholesterol, high-density lipoprotein cholesterol, total triglycerides, and glucose were determined in blood serum and hemoglobin in whole blood in all individuals before, during, and after the intake of ω 3 fatty acids. In pooled serum samples, the lipoprotein composition and the fatty acid composition of blood lipids were determined. All dosages caused a shift in the fatty acid composition of blood serum lipids in favor of ω 3 fatty acids and at the expense of ω 6 and/or ω 9 acids. In the sterol esters, only the percentage of C20:5 ω 3 increased. Maximum shifts depended on the amount of ω 3 acids ingested and were evident within 1 to 2 wk. Two wk after the last ingestion of ω 3 acids, the fatty acid composition of blood serum lipids had returned to the original state. In the groups receiving 8.2 g of ω 3 fatty acids, there was a significant decrease in serum triglyceride and very low-density lipoprotein levels, which is in accord with earlier observations. In the other parameters, including cholesterol and high-density lipoprotein cholesterol, no decrease or increase was observed. *Am. J. Clin. Nutr.* 34: 1752-1757, 1981.

KEY WORDS ω 3 fatty acids, fish oil, blood lipid composition

Introduction

In a cross-over diet study with 42 volunteers living in a monastery or in a convent (1), it was found that a fish diet as compared with a cheese diet alters a number of blood parameters. After 3 wk, serum triglycerides were considerably lower, serum cholesterol slightly lower and high-density lipoprotein (HDL) cholesterol slightly higher as compared to the period in which the subjects received cheese instead of fish. In the serum lipoproteins, a strong decrease in very low-density lipoproteins (VLDL) was observed and, in the fatty acid pattern of the serum lipids, there was a marked shift towards ω 3 fatty acids such as C20:5 ω 3 and C22:6 ω 3 at the expense of, in particular, C18:2 ω 6 and, to some extent, also of C18:1 ω 9. The daily ingestion of fish (mackerel containing 25% of fat) amounted to 200 g. From these data and from the fatty acid composition of mackerel fat, a daily intake of 8 g of ω 3 fatty acids was estimated. It was assumed that the changes observed were due to the presence of these fatty acids in the diet.

The results of this experiment stimulated us to perform further experiments in this area. First, the question arose as to whether the data obtained could be reproduced in other subjects living under other conditions. Second, it was thought important to know whether similar results could be produced through administration of lower dosages of ω 3 fatty acids. Since, in another experiment, it was demonstrated that large amounts of mackerel oil in the feed of piglets may induce a type of vitamin E deficiency known as "yellow fat disease" (2), it was believed that the intake of ω 3 fatty acids by man should not be higher than necessary for obtaining effects considered as beneficial.

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Therefore, an experiment was undertaken with the aim to not only reproduce the results already obtained but also to study the effect of lower doses of ω 3 fatty acids on blood serum composition. Some results of this experiment have been presented in a short communication (3).

Materials and methods

A ω 3 fatty acid concentrate was administered daily to the subjects during 4 wk. A concentrate instead of fish was used in order to exclude other fish components that might influence the parameters measured and to avoid the contribution of these components to the total fat intake. As in the previous experiment, vitamin E was supplied to all subjects in amounts of 100 mg on alternate days. The subjects were kept on their normal diets during the experiment.

They were divided into five groups. Four groups were administered ω 3 fatty acids in daily amounts of about 1, 2, 4, and 8 g, respectively; one group, in which the subjects received 1 g of olive oil daily, served as a control. Fasting venous blood samples were taken according to the time schedule presented in Table 1.

Subjects

The study group was recruited from the city of Groningen (population 165,000) and its suburbs by means of advertisements in local newspapers. The responders were selected according to the following criteria: age between 18 and 60 yr; good condition; use of no drugs; not on a diet regimen; serum cholesterol not exceeding 250 mg/100 ml; serum triglycerides lower than 200 mg/100 ml; consumption of fishery products not more than four times monthly.

The number of selected volunteers was 52. These were divided at random over the five groups. All groups consisted of 10 subjects except for the group in which the intake of

ω 3 fatty acids was greatest; in this group the number of subjects was 12.

The subjects were between 18 and 60 yr of age (mean 29.2 yr). The body weights of the men ranged from 58.7 to 85.0 kg (mean 76.9 kg) and those of the women from 51.3 to 74.7 kg (mean 62.4 kg). The heights ranged from 1.70 to 1.93 m for the men and from 1.56 to 1.84 m for the women (mean 1.82 and 1.70 m, respectively). None of the subjects showed a weight change of more than 2 kg during the intake of ω 3 fatty acids. All subjects kept a diary in which particulars with respect to health, irregularities in capsule intake, etc., were noted.

Preparation and administration of ω 3 fatty acids

The starting material was commercial cod liver oil. After saponification of the oil, the fatty acids were added to a hot saturated solution of urea in methanol in order to separate polyunsaturates from saturated and monounsaturated fatty acids. The latter form insoluble urea complexes and are removed by filtration. The procedure will be described in detail elsewhere (N. Haagsma, C. M. Van Gent, J. B. Luten, R. W. De Jong, and E. Van Doorn, manuscript in preparation).

The remaining fatty acids, 85% of which were ω 3 fatty acids,³ were extracted with hexane, provided with 0.01% of octyl gallate (on fatty acid base) and stored in hexane at -25°C under nitrogen in order to minimize oxidation and polymerization reactions.

A few days before administration, the hexane was removed by distillation under vacuum. The concentrate was packaged in gelatin capsules and stored at 0°C . Each capsule contained 0.525 g of fatty acid concentrate.

The number of capsules and the amount of ω 3 fatty acids administered to each group are given in Table 2. The fatty acid composition of the cod liver oil and of the concentrate is presented in Table 3.

Analysis of blood samples

The total serum cholesterol and triglycerides were determined by the method of Abell

³ As a result of the contact with methanol, one-third of the fatty acids were present as methyl esters. It was not considered necessary to prepare a new concentrate, thereby preventing this methylation; however, in future experiments ethanol should be preferred to methanol.

TABLE 1
Time schedule of the experiment

Day	0	11	18	25	32	39	53
	Intake of ω 3 fatty acids						
Determination of:							
Wt	x	x		x		x	
Ht		x					
Cholesterol	x	x	x	x	x	x	x
HDL cholesterol		x	x	x	x	x	x
Triglycerides	x	x	x	x	x	x	x
Glucose		x				x	x
Hb		x				x	x
Preparation of serum pools	x	x	x	x	x	x	x

TABLE 2
Group composition and $\omega 3$ fatty acid intake per group

Group	No. of subjects		No. of capsules daily	Amount of concentrate	Amount of $\omega 3$ fatty acids
	Men	Women			
I	4	6	2		
II	4	5	3	1.58	1.37
III	5	4	5	2.62	2.27
IV	5	5	9	4.72	4.09
V	6	5	18	9.45	8.19

TABLE 3
Fatty acid composition of cod liver oil and mean composition of batches of $\omega 3$ fatty acid concentrate

	Cod liver oil	Concentrate
C14:0	4.7	1.0
C16:0	11.4	
C16:1	9.1	1.3
C16:2	0.9	2.0
C18:0	2.2	
C18:1	24.9	2.0
C18:2	1.8	1.6
C18:3 $\omega 3$	1.0	1.0
C18:4 $\omega 3$	2.4	10.0
C20:1	12.0	
C20:3 $\omega 3$	0.1	0.7
C20:4 $\omega 3$		1.6
C20:5 $\omega 3$	12.1	27.6
C22:1	4.8	
C22:4		2.8
C22:5 $\omega 3$		1.2
C22:6 $\omega 3$	11.7	44.6
Σ	99.1	97.4
$\Sigma \omega 3$	27.3	86.7

et al. (5) and of Soloni (6), respectively. The HDL fraction was isolated following the method of the National Institutes of Health (7), the cholesterol content of which was determined according to Abell et al. (5). Glucose was determined by the procedure of Werner et al. (8) and hemoglobin by the method of Henry et al. (9). Pooled sera were prepared from 0.5 ml of serum from each subject of each group. The lipoprotein composition of the pooled sera was determined by the method of Van Gent (10). After thin-layer separation the fatty acid composition of serum lipid fractions was determined by GLC according to Woodford and Van Gent (11).

TABLE 4
Values for blood parameters in the different groups at the start (S) and the end (E) of $\omega 3$ fatty acid administration (mean and 95% confidence interval)

	I		II		III		IV		V	
	S	E	S	E	S	E	S	E	S	E
Cholesterol (mg/100 ml serum)	216 $\pm$ 16	215 $\pm$ 18	198 $\pm$ 24	201 $\pm$ 27	188 $\pm$ 27	194 $\pm$ 24	188 $\pm$ 22	193 $\pm$ 22	196 $\pm$ 26	195 $\pm$ 28
HDL cholesterol (mg/100 ml)	54 $\pm$ 5	54 $\pm$ 4	53 $\pm$ 11	54 $\pm$ 10	51 $\pm$ 10	54 $\pm$ 11	54 $\pm$ 11	53 $\pm$ 10	62 $\pm$ 9	65 $\pm$ 12
Triglycerides (mg/100 ml)	99 $\pm$ 21	109 $\pm$ 24	98 $\pm$ 27	83 $\pm$ 18	75 $\pm$ 16	72 $\pm$ 19	87 $\pm$ 23	86 $\pm$ 23	90 $\pm$ 18	55 $\pm$ 9
Glucose (mg/100 ml)	87 $\pm$ 3	85 $\pm$ 5	88 $\pm$ 9	86 $\pm$ 9	86 $\pm$ 4	87 $\pm$ 4	89 $\pm$ 7	86 $\pm$ 7	83 $\pm$ 4	83 $\pm$ 4
Hb (g/100 ml whole blood)	10.0 $\pm$ 0.6	10.3 $\pm$ 0.6	10.0 $\pm$ 0.5	10.2 $\pm$ 0.6	10.1 $\pm$ 0.5	10.3 $\pm$ 0.5	10.0 $\pm$ 0.5	10.1 $\pm$ 0.5	10.0 $\pm$ 0.5	10.2 $\pm$ 0.7

Results

On basis of the notes in the diaries, the observations in three subjects were taken out of consideration. For the remaining observations, Table 4 shows the mean values of the individual cholesterol, HDL cholesterol, triglyceride, glucose, and hemoglobin determinations for each group. As no significant differences were observed during the intake of ω 3 fatty acids, only the values at the start and at the end of ω 3 fatty acid administration are given. In Table 5, the lipoprotein composition of pooled sera is presented in the same manner.

Considerable changes were found in the concentration of serum triglycerides ($p < 0.01$) and in the VLDL fraction of serum lipoproteins in group V. For this reason, all values from this group involving these parameters are presented separately in Table 6.

Changes in fatty acid composition of serum lipids were observed in all groups with respect to the C18:1 ω 9, C18:2 ω 6, C20:5 ω 3, and C22:6 ω 3 acids. The values concerning these acids are presented in Table 7. The percentages of other fatty acids did not change significantly and are therefore omitted from Table 7; however, the values for C20:4 ω 6 are included to stress the absence of changes in the concentration of this fatty acid.

Discussion

The striking effect on serum triglycerides and VLDL in group V is in accord with our

observations on both human subjects (1) and piglets (2). This effect is not found in the other groups, which may indicate that only fairly large amounts of ω 3 fatty acids in the diet will cause a decrease in these parameters.

Both glucose and hemoglobin concentrations remained constant throughout the experiment, indicating that no adverse effects from the ω 3 fatty acid intake occurred.

Changes in fatty acid composition of serum lipids were found in all groups except for the control. The greatest changes were observed in group V and the smallest in groups II and III, indicating a dose-response effect. The maximum changes were observed after 1 and 2 wk. Two wk after the end of ω 3 fatty acid administration, all percentages returned to their original values.

Our previous observation that C22:6 ω 3 increases in all serum lipids except the sterol esters was confirmed by this experiment.

The conclusion can be drawn that the most important effect of short-term administration of ω 3 fatty acids on serum lipid composition is the shift in their fatty acid composition. The decrease in serum triglycerides and in VLDL, although interesting, was found to occur only at a daily intake of 8 g.

Contrary to the results obtained earlier by us (1), neither total serum cholesterol nor HDL cholesterol showed any change as a result of ω 3 fatty acid intake. This may indicate that the effect of ω 3 fatty acids on these parameters is occasional. In the previous experiment, however, the mean age of the sub-

TABLE 5
Lipoprotein composition of serum pools at the start (S) and the end (E) of ω 3 fatty acid administration

	I		II		III		IV		V	
	S	E	S	E	S	E	S	E	S	E
Chylomicrons (mg/100 ml)	14	13	13	7	6	11	12	18	6	12
VLDL mg/100 ml	123	111	106	78	82	71	124	101	112	52
LDL* (mg/100 ml)	364	386	368	386	300	278	302	339	305	356
HDL (mg/100 ml)	185	164	137	183	158	185	179	136	164	163

* Low-density lipoprotein.

TABLE 6
Changes in mean serum triglyceride content and in VLDL in group V in the course of the experiment

Day	0	11	18	25	32	39	53
Intake of ω 3 fatty acids							
Triglycerides (mg/100 ml)	70 $\pm$ 12	90 $\pm$ 18	72 $\pm$ 12	58 $\pm$ 10	59 $\pm$ 4	55 $\pm$ 9	81 $\pm$ 10
VLDL (mg/100 ml)		112	62	58	61	52	110

TABLE 7
Content of C18:1 ω 9, C18:2 ω 6, C20:4 ω 6, C20:5 ω 3, and C22:6 ω 3 fatty acids in serum lipid fractions in the course of the experiment*

Group	I										II										III										IV										V									
	11	18	25	32	39	53	11	18	25	32	39	53	11	18	25	32	39	53	11	18	25	32	39	53	11	18	25	32	39	53	11	18	25	32	39	53														
C18:1 ω 9 in:																																																		
Sterolesters	16	17	17	17	18	17	17	16	16	16	15	18	15	16	15	16	14	15	18	17	17	17	17	17	17	16	16	16	16	16	16	16	16	16	16	16	16	16	16											
Triglycerides	38	36	40	39	40	39	38	37	36	39	36	40	36	31	35	33	35	38	40	35	32	33	34	41	38	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30										
Phosphatidylcholine	12	12	12	12	12	11	11	11	11	11	11	12	10	11	11	11	11	12	11	11	11	10	11	12	11	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10								
C18:2 ω 6 in:																																																		
Sterolesters	59	56	59	57	56	59	53	56	56	56	57	55	58	54	57	58	56	59	58	49	51	52	53	58	58	50	49	50	50	50	50	50	50	50	50	50	50	50	50	50	50									
Triglycerides	19	23	19	19	18	20	20	21	21	20	23	17	21	22	22	23	23	21	20	16	17	19	21	18	21	18	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19								
Phosphatidylcholine	26	28	28	29	27	27	28	26	26	25	28	28	24	25	25	24	25	29	24	21	22	22	23	27	27	20	21	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20							
C20:4 ω 6 in:																																																		
Sterolesters	5	5	5	6	6	6	6	6	6	6	6	6	6	7	7	7	7	6	6	7	7	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7								
Triglycerides	1	1	1	1	1	1	1	1	1	2	1	2	1	2	2	1	1	1	1	1	1	1	1	1	1	0	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1							
Phosphatidylcholine	7	8	8	8	7	8	8	8	8	8	7	7	9	10	9	8	8	8	8	9	9	8	7	8	9	8	9	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7							
C20:5 ω 3 in:																																																		
Sterolesters	0	1	1	1	1	1	1	1	2	3	3	3	1	1	2	3	3	4	1	1	4	5	6	5	1	1	8	8	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9						
Triglycerides	0	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	2	1	0	0	1	3	2	2	0	0	5	6	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5						
Phosphatidylcholine	1	1	0	0	1	1	1	2	2	2	2	1	1	2	2	2	3	1	1	3	5	4	3	1	1	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7						
C22:6 ω 3 in:																																																		
Sterolesters	0	1	0	1	1	0	0	1	1	2	1	1	1	0	1	1	1	1	1	0	1	2	1	1	1	0	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
Triglycerides	0	1	0	2	1	0	0	2	3	3	2	1	0	2	3	3	3	3	1	0	4	6	4	3	1	0	8	10	9	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8						
Phosphatidylcholine	3	3	3	3	3	3	3	2	5	6	6	4	3	2	5	5	6	6	3	3	6	7	8	6	4	2	7	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8						

* The period of ω 3 fatty acid intake is during days 18-30.

* The period of ω 3 fatty acid intake is during days 18 to 39.

jects (46 yr) was considerably higher than in the experiment described here. Furthermore, it is worth mentioning that the Eskimo diet, containing large amounts of ω 3 fatty acids, does not lead to extremely low serum cholesterol values (12).

The changes in serum fatty acid composition may be of much significance in the reduction of thrombocyte aggregation. Dyerberg et al. (13) have suggested that a high C20:5 ω 3/C20:4 ω 6 ratio in plasma may lead to a state indicated as "antithrombotic," in which the antiaggregating prostacyclin PGI₂ and the nonactive thromboxane TXA₂, both from C20:5 ω 3, are of importance (13).

However, C20:5 ω 3 has been found to be only a relatively poor substrate for platelet cyclooxygenase, being converted only one-eighth as efficient as C20:4 ω 6 to thromboxane (14). A similar observation was made by Hornstra et al. (15). Thus, the effect of C20:5 ω 3 on thrombocyte aggregation seems to be not only that of a replacement of PGI₂ by PGI₃ and of TXA₂ by TXA₃, but also that of a reduction of prostacyclin and thromboxane synthesis in general.

In recent studies the effect of ω 3 fatty acid intake on the C20:5 ω 3/C20:4 ω 6 ratio and on thrombocyte aggregation is demonstrated again. Siess et al. (16), after administration of large amounts of mackerel to volunteers, observed a marked increase of this ratio in both plasma and platelet phospholipids after 6 days. In addition, they noted simultaneous reductions in TXA₂ formation and platelet aggregation after collagen stimulation in platelet-rich plasma. Sanders et al. (17), in an experiment with 12 subjects, administered 20 g of cod liver oil daily for 6 wk and also found an increase in the C20:5 ω 3/C20:4 ω 6 ratio in the fatty acids of platelet phospholipids, accompanied by a moderate but highly significant increase in bleeding time. Their design was comparable, to some extent, with that of our group IV. This experiment also indicates that no excessive amounts of ω 3 fatty acids are necessary to cause effects that might reduce the risk for ischemic cardiovascular diseases.

Our previous observation that C22:6 ω 3 increases in all serum lipids except the sterol esters was confirmed by this experiment. Explanations for this phenomenon are not more than speculative.

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Retroconversion and metabolism of [^{13}C]22:6n-3 in humans and rats after intake of a single dose of [^{13}C]22:6n-3-triacylglycerols¹⁻³

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ABSTRACT The apparent retroconversion of docosahexaenoic acid (22:6n-3) to eicosapentaenoic acid (20:5n-3) and docosapentaenoic acid (22:5n-3) was studied in vivo, in rats and humans, after they ingested a single dose of triacylglycerols containing [^{13}C]22:6n-3 ([^{13}C]22:6-triacylglycerol), without 22:6n-3 dietary supplementation. The amount of apparent retroconversion and the distribution of the three n-3 polyunsaturated fatty acids (PUFAs) in plasma lipid classes were followed as a function of time by measuring the appearance of ^{13}C in these PUFAs with gas-chromatography combustion-isotope ratio mass spectrometry. This [^{13}C]22:6n-3 retroconversion, calculated by summing the amounts of [^{13}C]22:5n-3 and [^{13}C]20:5n-3 in plasma lipids, was lower in humans than in rats, reaching a maximum of $\approx 9\%$ of the total plasma [^{13}C]22:6n-3 in rats, but only 1.4% in humans. The incorporation of [^{13}C]22:6n-3 and [^{13}C]22:5n-3 in lipid classes followed their endogenous distribution with a maximal accumulation in phospholipids, but a low incorporation into cholesterol esters (CEs), whereas [^{13}C]20:5n-3 was equally present in phospholipids and CEs. The ratio of the amount of HDL-CE to HDL-phosphatidylcholine for [^{13}C]20:5n-3 was higher than for [^{13}C]22:6n-3, indicating a selectivity of the lecithin-cholesterol acyltransferase enzyme with regard to these PUFAs, which may be related to the differences in their biological properties after fish oil feeding. The occurrence of a weak basal 22:6n-3 retroconversion in humans supports feeding this pure PUFA in cases in which 20:5n-3 presents undesirable side effects and when specific alterations of blood lipids are expected. *Am J Clin Nutr* 1996;64:577-86.

KEY WORDS Docosahexaenoic acid, eicosapentaenoic acid, retroconversion, n-3 polyunsaturated fatty acids, lecithin-cholesterol acyltransferase, stable isotope and gas-chromatography combustion-isotope ratio mass spectrometry

INTRODUCTION

n-3 Polyunsaturated fatty acids (PUFAs) represent an essential fatty acid family, the precursor being α -linolenic acid (18:3n-3). The desaturation and elongation of 18:3n-3 leads to the formation of eicosapentaenoic (20:5n-3), docosapentaenoic (22:5n-3), and docosahexaenoic (22:6n-3) acids. 22:6n-3 is the unique n-3 PUFA highly represented in phospholipids of the brain gray matter and photoreceptor outer

segments of the retina (1). Its depletion in these tissues by dietary deficiency during periods of growth and development induces functional impairments, such as limited visual acuity and altered physiologic responses of the retina (2). On the other hand, n-3 PUFAs are found in high concentrations in fish and marine food and their concentrations increase in plasma lipids in fish oil feeding experiments. These changes induce anti-atherogenic and antithrombotic effects in animals and humans by modulating the nature and concentrations of blood lipids and lipoproteins and by fatty acid incorporation into blood cell lipids, in which they inhibit eicosanoid formation (3). Because fish oil contains both 20:5n-3 and 22:6n-3, the respective role of these fatty acids in the effects observed is not elucidated (4). Knowledge of their specific metabolism in plasma lipids and lipoproteins, in relation to their relative biological potencies is necessary to target n-3 dietary supplements for specific beneficial effects. Feeding pure 20:5n-3 leads to the formation of other n-3 long-chain PUFAs by elongation and desaturation, but feeding pure 22:6n-3 leads to the sole accumulation of this PUFA because retroconversion is a minor metabolic pathway (5). Retroconversion is a peroxisomal reaction, involving one cycle of β -oxidation with $\Delta 4$ enoyl CoA reductase and $\Delta 3$, $\Delta 2$ enoyl CoA isomerase as auxiliary enzymes (6). Its regulation, particularly its quantitative importance in vivo, is not known. In cultured cells, the retroconversion of long-chain fatty acids seems to be stimulated by the mass effect of substrates (7). Feeding high concentrations of pure 22:6n-3 as ethyl esters induced an accumulation of 20:5n-3 in human plasma phospholipid (8). However, the basal 22:6n-3 retroconversion occurring in nonsupplemented subjects, or in subjects fed low amounts of 22:6n-3, has never been quantified. During a low supply of pure 22:6n-3 to formula-fed babies who are deficient in this fatty acid is of interest because

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20:5n-3 may have adverse effects in these babies (9) by replacing arachidonic acid and thereby inducing growth impairment (10). In addition, plasma lipid and lipoprotein indexes respond differently to the 20:5n-3- and 22:6n-3-rich oil (4), probably resulting from the preferential incorporation of these n-3 PUFAs in transport plasma lipids (11-13) and from their specific reactivity toward lipid metabolism enzymes (11, 14).

These observations were reported after ingestion of relatively large amounts of fish oil, varying greatly in the concentration of the two main n-3 PUFAs and other fatty acids, which may indirectly influence the specific lipid metabolism of these n-3 PUFAs. For these reasons, the metabolic fate of 22:6n-3, 22:5n-3, and 20:5n-3 was traced in vivo by using 22:6n-3 labeled with the stable isotope carbon-13 (^{13}C), and were detected by gas-chromatography combustion-isotope ratio mass spectrometry (GCC-IRMS) (15). After ingestion of a unique dose of triacylglycerols rich in [^{13}C]22:6n-3 ([^{13}C]22:6-triacylglycerol), the apparent retroconversion of 22:6n-3 was quantified in plasma and the divergences of lipid metabolism of 22:6n-3 and of its retroconverted products were studied without dietary 22:6n-3 supplementation in humans and rats.

SUBJECTS, MATERIALS, AND METHODS

Triacylglycerols containing [^{13}C] docosahexaenoic acid were produced by growing a microalgae strain in a defined medium containing 16.7 mmol D-[1- ^{13}C]glucose/L (Eurisotop, St Aubin, France). After the cells were harvested by centrifugation (1000 $\times$ g, 10 min, 4 $^{\circ}\text{C}$), the biomass was freeze-dried.

For the rat experiment, total lipids were extracted according to the method of Bligh and Dyer (16). Triacylglycerols, which represented 65% of total lipids, were purified by thin-layer chromatography (TLC) using silica gel 60 plates (Merck, Darmstadt, Germany) and hexane:ether:acetic acid mixture (90:30:1, by vol) as the developing solvent. These total triacylglycerols [retardation factor(Rf): 0.67] were extracted from the silica gel with chloroform:methanol (90:10, by vol).

For the human experiment, total lipids were extracted with isopropanol:hexane (3:2, by vol) and triacylglycerol purification was carried out on an alumina column (20 $\times$ 1 cm) containing 10 g 5% deactivated alumina in water. Two grams of oil was dissolved in 20 mL hexane and the triacylglycerols were eluted from the column, under nitrogen, by adding 50 mL hexane three successive times. The fatty acid composition of these triacylglycerols is reported in Table 1. [^{13}C]22:6n-3

TABLE 1
Fatty acid composition of [^{13}C]22:6n-3-triacylglycerol

Fatty acid	Amount ¹
	mol %
10:0	0.8
12:0	2.4
14:0	13.6
16:0	21.6
16:1	1.3
18:0	0.6
18:1	15.0
22:6	44.4

¹ Mol % of total fatty acids.

represented 44 mol % of total fatty acids and had an abundance of ^{13}C of 7.72 atom %. [^{13}C]22:5n-3 and [^{13}C]20:5n-3, which remained undetectable by GC analysis without overloading the GC capillary column, were analyzed after HPLC preparation. The fatty acid methyl esters obtained from 1 mg of these [^{13}C]22:6-triacylglycerols were injected on an HPLC column (17) and [^{13}C]22:5n-3 and [^{13}C]20:5n-3 were collected. They were further quantified by GC analysis with 20:4n-6 methyl ester as an internal standard. They represented < 0.01 mol % of the total fatty acids in the tracer triacylglycerol.

Rat feeding

Rat experiments were performed in accordance with French and European Economic Community (86:609:EEC) guidelines for care and use of laboratory animals. Male Sprague-Dawley rats (300 g) were fed a standard laboratory starch diet (Souriffarat; Genthon, Chassieu, France). The triacylglycerol tracer was taken to dryness under nitrogen and immediately dispersed into 600 μL skim milk (lipids: 0.24 g/L; proteins: 2.9 g/L; carbohydrates: 4.4 g/L) for rat intubation. The triacylglycerol was adequately redissolved, as ascertained after Bligh and Dyer (16) extraction of aliquots of this solution. After food was withheld from the animals overnight, each rat was given 3 mg [^{13}C]22:6-triacylglycerol in 600 μL skim milk by intragastric intubation and then were given free access to a water solution containing 110 mmol glucose/L, 85 mmol NaCl/L, and 1.34 mmol KCl/L (wt:vol). Four to five rats were used for each time point, and five rats received only 600 μL skim milk. Food was withheld from rats overnight before they were killed but they were refed 12 h after tracer ingestion. At periods of 3, 6, 12, 18, 48, and 72 h after tracer ingestion, animals were anesthetized by peritoneal injection of pentobarbital and 10 mL blood was taken via cardiac puncture into chilled plastic syringes containing ACD: 38 mmol citric acid/L, 68 mmol citrate/L, and 136 mmol dextrose/L (pH 4.5) as anticoagulant.

Human feeding

The scientific protocol was accepted by INSERM and the local ethical committee. Three healthy subjects who had fasted overnight ingested 280 mg tracer triacylglycerol mixed in yogurt (lipids: 3.5%; proteins: 3.7%; carbohydrates: 5%) and had a breakfast immediately after this. At various times after ingestion, blood was taken by venipuncture into chilled plastic syringes containing ACD, as anticoagulant. A blood sample was also collected just before tracer ingestion to measure the basal ^{13}C abundance in lipid pools.

Plasma and blood cell preparation

All experimental steps were performed at 4 $^{\circ}\text{C}$. Blood samples were immediately centrifuged at 100 $\times$ g for 15 min to obtain platelet-rich plasma (PRP). PRP was centrifuged at 1000 $\times$ g for 10 min and the supernate was treated for lipoprotein preparation. The resulting platelet pellet was rinsed twice and gently resuspended in a Tyrode/HEPES buffer (135 mmol NaCl/L, 2.68 mmol KCl/L, 0.46 mmol NaHPO₄/L, 1 mmol MgCl₂/L, 5 mmol HEPES/L, 5.5 mmol glucose/L, and 1 mmol EDTA/L, pH 6.4) (18). Red cells were prepared as described previously (18).



High-density-lipoprotein isolation

The total fraction of HDL was prepared by ultracentrifugation at $600\,000 \times g$ in a fixed-angle rotor (TFT 80.49; Kontron, Zurich) for 2 h at 4 °C, with a sodium chloride:potassium bromide density gradient (15).

Fractionation and quantification of lipids

Total lipids were extracted from plasma, HDL, and blood cells according to Bligh and Dyer (16). Plasma lipid classes were prepared by spotting the lipid extract on silica gel 60 plates and developing total phospholipids (Rf: 0.0), triacylglycerols (Rf: 0.58), and cholesterol esters (CEs) (Rf: 0.94) with hexane:diethyl ether:acetic acid (80:20:1, by vol) as a mobile phase. Phosphatidylcholine (Rf: 0.47) and neutral lipids from HDL were purified on TLC plates with chloroform:methanol:water (65:25:4, by vol) as the mobile phase. CEs from HDL were prepared by a second separation of neutral lipids from HDL, with hexane:diethyl ether:acetic acid (80:20:1, by vol). Platelet and red blood cell phospholipid classes were purified by TLC migration with chloroform:methanol:14% aqueous methylamine (60:20:5, by vol) as a mobile phase, after predeveloping the plate with chloroform:methanol (80:8, by vol). Lipid classes were scraped off the plate and treated for 90 min with 5% H₂SO₄ in methanol to obtain fatty acid methyl esters. Lipid classes were quantified by adding internal standards in the extraction mixture. Triheptadecanoyl glycerol, heptadecanoyl-cholesterol, and 1,2-diheptadecanoyl-glycero-3-phospho-choline and -ethanolamine were added when appropriate and the absolute amounts of fatty acid methyl esters were determined by GC analyses, relative to the known amount of added 17:0 (19). Fatty acid methyl esters were analyzed and quantified by GC by using an SP 2380 capillary column (30 m $\times$ 0.32 mm) (Supelco, Bellefonte, PA) (20). Before their GCC-IRMS analysis, fatty acid methyl esters were purified by HPLC with a 5- μ m Superspher 100 C₁₈ column (Merck, Darmstadt, Germany) and by isocratic elution with acetonitrile:water (80:20, by vol) (15). They were detected by ultraviolet absorbance at 210 nm, collected, and the different fractions rapidly taken to dryness under nitrogen.

Gas-chromatography combustion-isotope ratio mass spectrometry analyses

Samples were injected onto an HP 5890 gas chromatograph equipped with a Ross injector and a Supelco capillary column (SP-2380, 0.25 mm $\times$ 30 m). The temperature gradient was held at 160 °C for 1 min and raised to 230 °C at 20 °C/min, at which it was maintained for 5 min. A heart-cut valve directed the helium flow to either the flame-ionization detector or to a quartz tube filled with cupric oxide, heated at 800 °C, where carbon dioxide was generated from fatty acids by catalytic combustion. The effluent was then driven into a water trap before its ionization by electron impact in the source of the isotope-ratio mass spectrometer (SIRA 12; VG Isogas, Middleswich, United Kingdom). Fatty acid oxidation and water trapping was complete, as reported previously (21). The different isotopomers were collected onto three different collectors at *m/z* 44, 45, and 46. Before and after the carbon dioxide peak generated from the fatty acids, a carbon dioxide reference sample of known enrichment calibrated against the international standard (Pee Dee Belemnite; PDB) was automatically

injected into the mass spectrometer (15). The ¹³C/¹²C values of the samples and the reference were used to calculate the δ per 1000 value ($\delta^{13}\text{C}_{\text{‰}}$) with the following equation:

$$\delta^{13}\text{C}_{\text{‰}} = \frac{{}^{13}\text{C}/{}^{12}\text{C sample} - {}^{13}\text{C}/{}^{12}\text{C reference}}{{}^{13}\text{C}/{}^{12}\text{C reference}} \times 1000 \quad (1)$$

where the reference is the international PDB standard, which is carbon dioxide obtained from the carbonate shell of a cretaceous mollusk. The relative contribution of the derivative carbon to the $\delta^{13}\text{C}_{\text{‰}}$ values for 22:6n-3, 22:5n-3, and 20:5n-3 was corrected according to the method of Khalfallah et al (22). Atom % values are related to $\delta^{13}\text{C}_{\text{‰}}$ as described (15). The appearance of [¹³C]22:6n-3, [¹³C]22:5n-3, and [¹³C]20:5n-3 in biological samples was also expressed as the absolute amounts of [¹³C]fatty acids in the lipid pools, by determination of [¹³C]fatty acid dilution with standard curves and by multiplying these values by the endogenous 22:6n-3, 22:5n-3, and 20:5n-3 concentrations, measured by GC (19).

Statistical analysis

The basal ¹³C abundances of n-3 fatty acids in human and rat lipids were compared by using Student's *t* test.

RESULTS

Natural ¹³C abundance of n-3 fatty acids

The natural ¹³C content of 20:5n-3, 22:5n-3, and 22:6n-3 was analyzed in human and rat lipid classes to find out whether an extensive interconversion of these fatty acids in vivo, by desaturation and elongation or retroconversion, led to the homogeneity of their ¹³C abundance. In humans, the mean ($\pm$ SD) baseline value of [¹³C]22:6n-3 was $-26.96 \pm 0.55\text{‰}$ and differed significantly from the values of 20:5n-3 ($-30.59 \pm 0.65\text{‰}$, $P < 0.0001$) and 22:5n-3 ($-29.76 \pm 0.89\text{‰}$, $P < 0.0001$; Student's *t* test; $n = 15-25$). In rats, these values were $-25.62 \pm 0.53\text{‰}$ for 22:6n-3, $-28.99 \pm 1.04\text{‰}$, and $-28.55 \pm 0.69\text{‰}$ for 20:5n-3 and 22:5n-3, respectively (significantly different from 22:6n-3, $P < 0.0001$, $n = 25-45$).

Retroconversion amounts of [¹³C]22:6n-3 in humans and rats

The retroconversion of [¹³C]22:6n-3 was calculated by summing the amounts of [¹³C]20:5n-3 and [¹³C]22:5n-3 measured in plasma triacylglycerols, phospholipids, and CEs as a function of time after ingestion (Figure 1). The retroconversion took place in the liver and the amounts of [¹³C]22:5n-3 and [¹³C]20:5n-3 in the plasma represented an apparent retroconversion. The overall amounts of the retroconversion products was higher in rat than in human plasma. These products were detected very early in plasma, as early as 1 h after ingestion in humans, but the rate of conversion appeared to be more rapid in rats than in humans. The amounts of [¹³C]20:5n-3 and [¹³C]22:5n-3 produced did not differ significantly in humans, whereas the synthesis of [¹³C]20:5n-3 was two to three times that of [¹³C]22:5n-3 in rats for each time point. [¹³C]20:5n-3 and [¹³C]22:5n-3 labeling was not influenced by desaturation and elongation of [¹³C]18:3n-3, which was not detected in the tracer triacylglycerol because the labeling



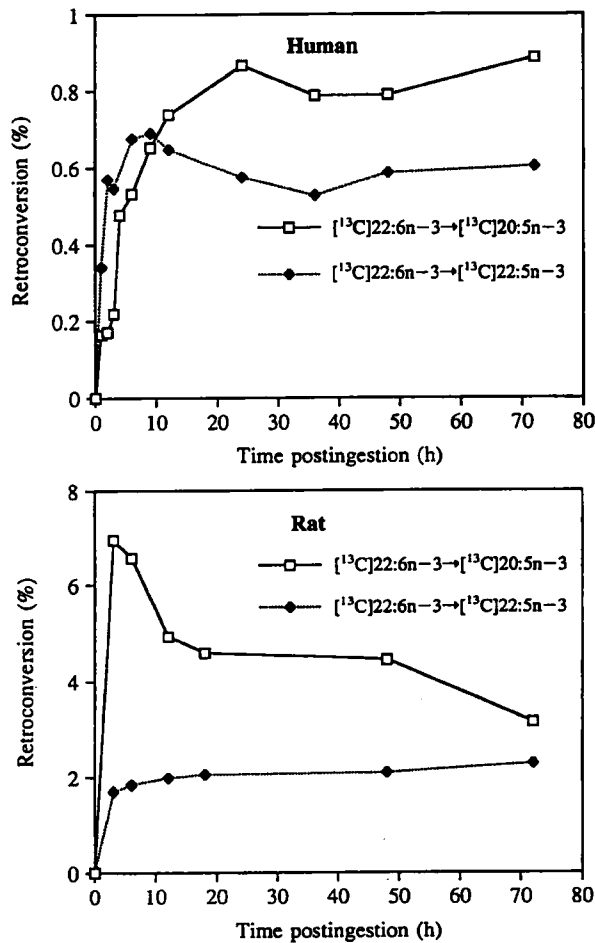


FIGURE 1. Percentage of plasma $[^{13}\text{C}]22:6n-3$ converted to $[^{13}\text{C}]20:5n-3$ and $[^{13}\text{C}]22:5n-3$ in humans and rats, as a function of time after ingestion of a tracer dose of $[^{13}\text{C}]22:6$ -triacylglycerol.

kinetics of this fatty acid in several rat and human lipid classes did not differ from baseline (not shown). Moreover, a ^{13}C enrichment of $20:5n-3$ and $22:5n-3$ occurring through the oxidation of the tracer $[^{13}\text{C}]$ fatty acids followed by the $[^{13}\text{C}]$ acetyl CoA recycling was negligible or null because $18:3n-3$, $18:3n-6$, $18:2n-6$, $20:4n-6$, and $22:4n-6$ were not labeled in rat and human lipids.

Time course for appearance of $[^{13}\text{C}]22:6n-3$ in plasma lipids

The appearance of $[^{13}\text{C}]22:6n-3$ was followed as a function of time over a period of 72 h in plasma triacylglycerols, phospholipids, and CEs in humans and rats after a single ingestion of a tracer dose of $[^{13}\text{C}]22:6$ -triacylglycerol (Figure 2). The maximal enrichment observed in triacylglycerol was at 3 and 2 h postigestion in rats and humans, respectively. The $22:6n-3$ $\delta^{13}\text{C}\text{‰}$ values in rats increased from $-24.84 \pm 0.18\text{‰}$ to $+331.87 \pm 63.90\text{‰}$, at 3 h, which was the earliest time point studied. The $\delta^{13}\text{C}\text{‰}$ values were higher in humans than in rats at 2 h, and rose from $-27.66 \pm 0.58\text{‰}$ to $+2145 \pm 869\text{‰}$. The ^{13}C abundance sharply declined until 12 h and plateaued until 3 d, where it remained at positive $\delta^{13}\text{C}\text{‰}$ values

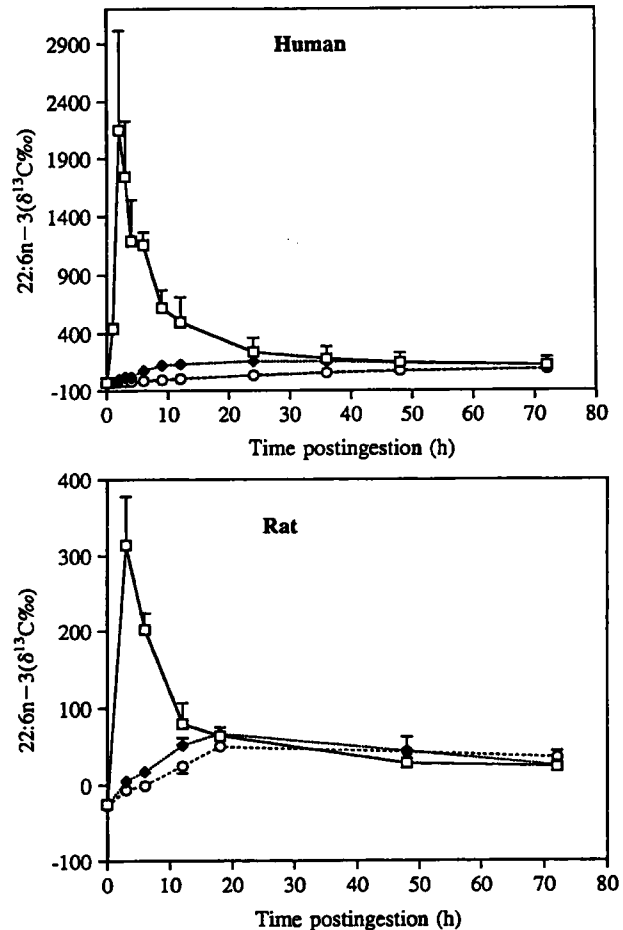


FIGURE 2. Time course of $[^{13}\text{C}]22:6n-3$ in plasma triacylglycerols ($\square$), phospholipids ($\blacklozenge$), and cholesterol esters ($\circ$) in humans and rats as a function of time after ingestion of a tracer dose of $[^{13}\text{C}]22:6$ -triacylglycerol. Each point represents the $\bar{x} \pm \text{SD}$ of three human subjects and the $\bar{x} \pm \text{SEM}$ of three to five rats.

($+23.38 \pm 9.30\text{‰}$ and $+117.55 \pm 72\text{‰}$ in rats and humans, respectively, at 72 h). Concomitant with the decline of triacylglycerol labeling, the ^{13}C abundance increased progressively in total phospholipids and CEs; reached a maximum of $+66.50 \pm 7.37\text{‰}$ and $+49.64 \pm 7.00\text{‰}$, respectively, at 18 h in rats, and remained close to this value 3 d postigestion. In humans, the labeling was higher in phospholipids than in CEs and peaked 24 and 72 h after ingestion, respectively.

The amount of $[^{13}\text{C}]22:6n-3$ incorporated in plasma lipid classes was calculated from their ^{13}C abundance and from their $22:6n-3$ endogenous content (Table 2). The distribution of endogenous $22:6n-3$ within these three lipid classes was different in rats and humans. In rats, this PUFA was equally present in triacylglycerol and phospholipid, whereas it was mainly associated with phospholipids in humans (Table 2). Eighty-nine percent of the incorporated $[^{13}\text{C}]22:6n-3$ was esterified in triacylglycerol at 2 and 3 h in humans and rats, respectively, whereas 12 h after ingestion and later, $[^{13}\text{C}]22:6n-3$ circulated in higher amounts in phospholipid (Figure 3). At this time, the esterification in phospholipid was quantitatively more important in humans than in rats, with 74% com-

TABLE 2

Distribution of endogenous 22:6n-3 within human and rat plasma lipid classes¹

Plasma lipid classes	Humans	Rats
	$\mu\text{mol/L}$	
Triacylglycerols	12.8 ± 8.2	56.7 ± 3.6
Phospholipids	111.6 ± 33.5	65.2 ± 14.9
Cholesterol esters	16.5 ± 4.0	16.8 ± 1.2

¹ $\bar{x} \pm \text{SD}$ of three human subjects and the $\bar{x} \pm \text{SEM}$ of five rats.

pared with 42% of the total plasma [^{13}C]22:6n-3 present in phospholipid, respectively. Only a small amount was esterified in cholesterol (9.6% in humans at 72 h and 10.9% in rats at 18 h). Three days after its ingestion, [^{13}C]22:6n-3 circulated mainly esterified in phospholipid (82%) in humans, whereas in rats it was associated with triacylglycerols and phospholipids (39% and 47% of total plasma [^{13}C]22:6n-3, respectively).

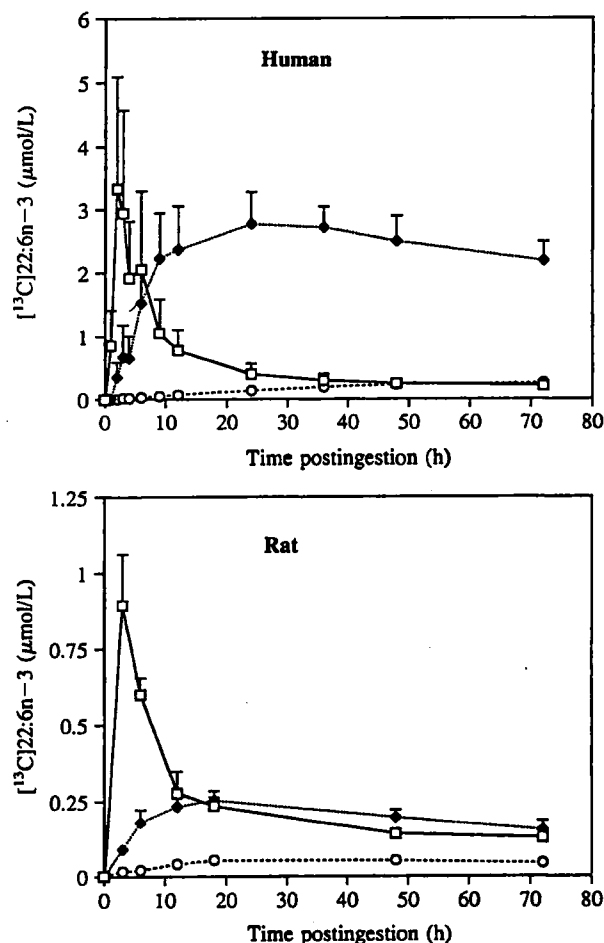


FIGURE 3. Concentration of [^{13}C]22:6n-3 in plasma triacylglycerols ($\square$), phospholipids ($\blacklozenge$), and cholesterol esters ($\circ$) in humans and rats as a function of time after ingestion of a tracer dose of [^{13}C]22:6-triacylglycerol. The amount of [^{13}C]22:6n-3 in each lipid class was calculated from $\delta^{13}\text{C}_r$ values and from endogenous 22:6n-3 concentrations. Each point represents the $\bar{x} \pm \text{SD}$ of three human subjects and the $\bar{x} \pm \text{SEM}$ of three to five rats.

Time course for appearance of [^{13}C]22:5n-3 in plasma lipid classes

Labeling of 22:5n-3, increasing as a function of time after tracer ingestion, was detected in triacylglycerols and phospholipids from humans and rats (Figure 4). The amount of endogenous 22:5n-3 in CEs was so weak that its ^{13}C abundance was not measured. In humans, the ^{13}C abundance of 22:5n-3 increased rapidly in plasma triacylglycerol and was significantly different from baseline as early as 1 h postingestion. Maximal labeling was observed between 2 and 6 h postintake (about -12‰). This labeling decreased until 3 d but did not return to baseline ($-26.67 \pm 1.67\text{‰}$ at 72 h compared with $-30.29 \pm 0.83\text{‰}$ at baseline). The appearance of [^{13}C]22:5n-3 in phospholipid was more progressive, increasing from $-30.46 \pm 0.11\text{‰}$ at baseline to $-26.79 \pm 0.72\text{‰}$ at 72 h (Figure 4). In rats, the magnitude of variation was higher. The ^{13}C abundance of 22:5n-3 in triacylglycerols increased from $-28.58 \pm 0.22\text{‰}$ to $+14.21 \pm 10.61\text{‰}$ 3 h postingestion and from $-28.37 \pm 0.18\text{‰}$ to $-16.22 \pm 2.03\text{‰}$ in phospholipids at 18 h. Three days after intake, 22:6n-3 was still enriched

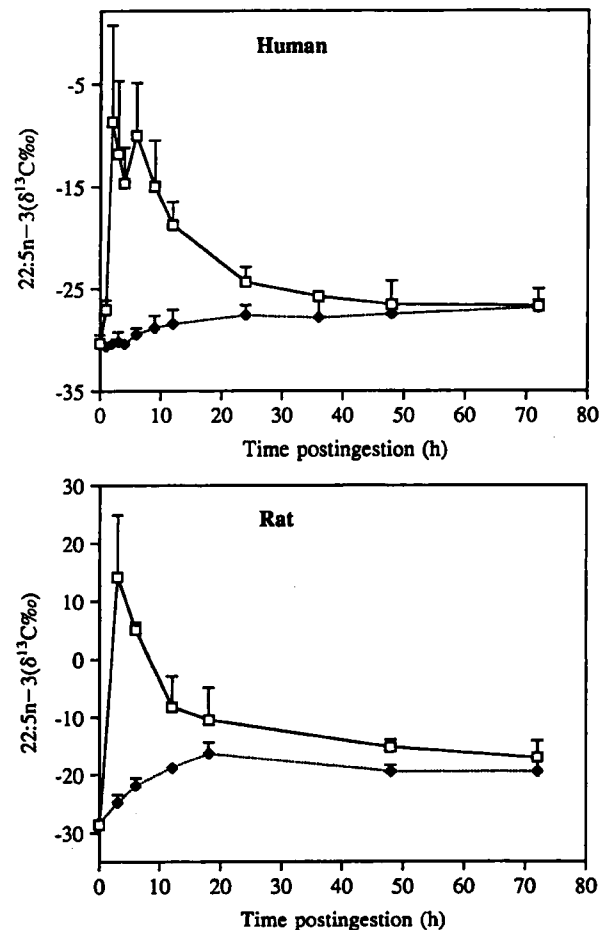


FIGURE 4. Time course of [^{13}C]22:5n-3 in plasma triacylglycerols ($\square$) and phospholipids ($\blacklozenge$) in humans and rats as a function of time after ingestion of a tracer dose of [^{13}C]22:6-triacylglycerol. Each point represents the $\bar{x} \pm \text{SD}$ of three human subjects and the $\bar{x} \pm \text{SEM}$ of three to five rats.



($-17.02 \pm 2.98\%$ and $-19.58 \pm 1.89\%$ in triacylglycerol and phospholipid, respectively) (Figure 4).

The [^{13}C]22:5n-3 concentration was higher in human triacylglycerol than in phospholipid, only until 12 h after ingestion, whereas after this, most of the [^{13}C]22:5n-3 was located in phospholipids (Figure 5). In contrast, in rats it was higher in triacylglycerols than in phospholipids for the entire period; 3 h after intake, triacylglycerol contained 12 times the amount of [^{13}C]22:5n-3 in phospholipid (48 compared with 4 nmol/L plasma).

Time course for appearance of [^{13}C]20:5n-3 in plasma lipid classes

In humans, the maximal labeling of 20:5n-3 occurred in plasma triacylglycerols 6 h after ingestion ($-17.28 \pm 3.64\%$) and it decreased until 72 h (Figure 6). The esterification of [^{13}C]20:5n-3 in phospholipid increased progressively until 12 h and plateaued over the 12-72-h period. The weakest labeling was observed in plasma CEs, where the [^{13}C]20:5n-3 appearance increased linearly as a function of time with the

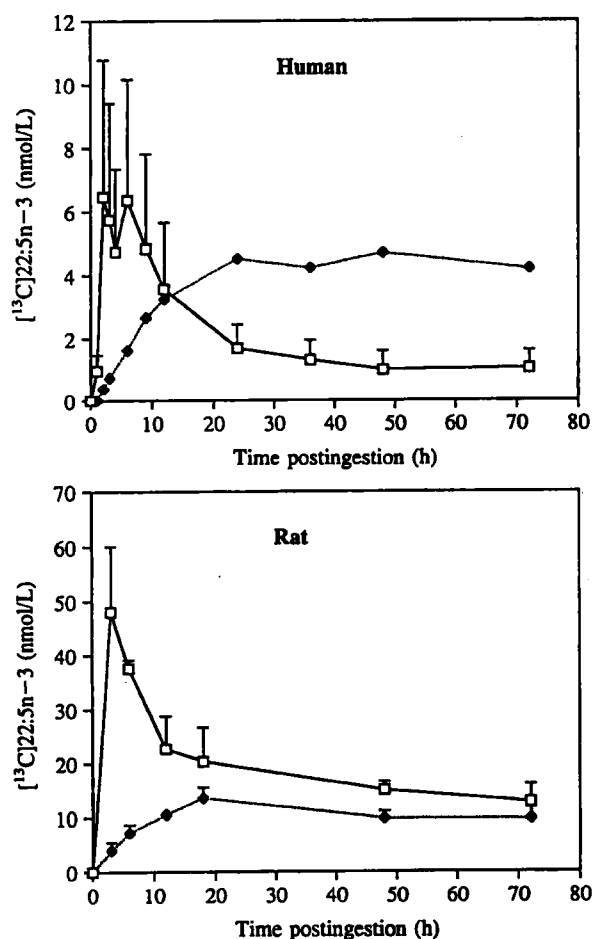


FIGURE 5. Concentration of [^{13}C]22:5n-3 in plasma triacylglycerols (□) and phospholipids (◆) in humans and rats as a function of time after ingestion of a tracer dose of [^{13}C]22:6-triacylglycerol. The amount of [^{13}C]22:5n-3 in each lipid class was calculated from $\delta^{13}\text{C}\%$ values and from endogenous 22:5n-3 concentrations. Each point represents the $\bar{x} \pm \text{SD}$ of three human subjects and the $\bar{x} \pm \text{SEM}$ of three to five rats.

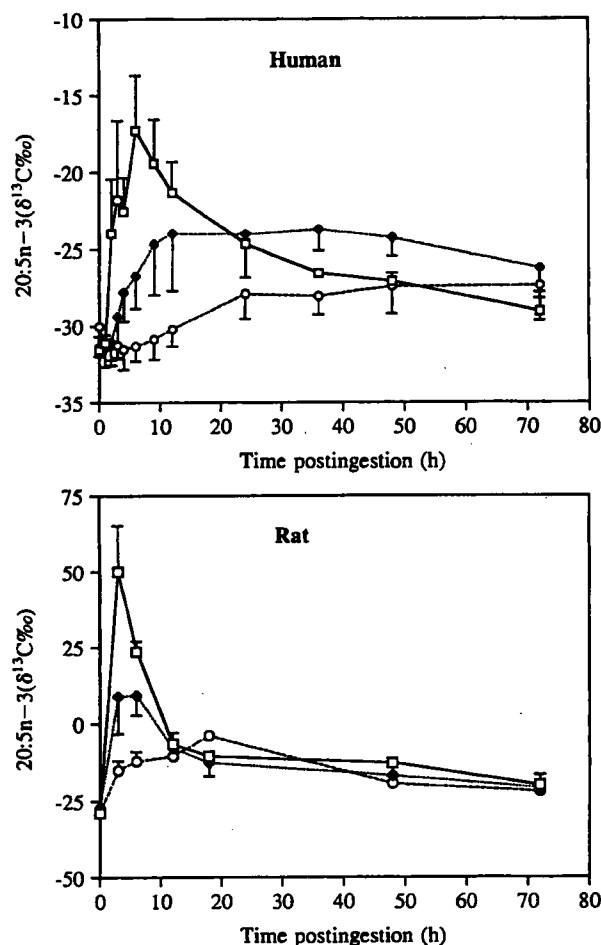


FIGURE 6. Time course of [^{13}C]20:5n-3 in plasma triacylglycerols (□), phospholipids (◆), and cholesterol esters (○) in humans and rats as a function of time after ingestion of a tracer dose of [^{13}C]22:6-triacylglycerol. Each point represents the $\bar{x} \pm \text{SD}$ of three human subjects and the $\bar{x} \pm \text{SEM}$ of three to five rats.

highest $\delta^{13}\text{C}\%$ value at 72 h post-ingestion. As for [^{13}C]22:5n-3, higher labeling was observed in rats than in humans. In rat phospholipids, [^{13}C]20:5n-3 appeared rapidly with a maximum at 3-6 h, followed by a decline and a plateau over the 12-72-h period (Figure 6).

The distribution of [^{13}C]20:5n-3 within lipid classes was different between humans and rats (Figure 7). At 12 h, [^{13}C]20:5n-3 was mainly esterified in rat triacylglycerol (64% compared with 19% and 16% in phospholipids and CEs, respectively), whereas it was located in CEs and phospholipids in humans (51% and 42%, respectively compared with 7% in triacylglycerol).

Appearance of retroconversion products in human blood cells

The appearance of [^{13}C]20:5n-3 and [^{13}C]22:5n-3 in platelet phosphatidylcholine increased from baseline values of $-30.51 \pm 0.31\%$ and $-29.43 \pm 0.53\%$, respectively, to $-26.39 \pm 0.47\%$ and $-27.56 \pm 0.93\%$, respectively, at 12 h, and plateaued until 72 h (Figure 8). The concentration of

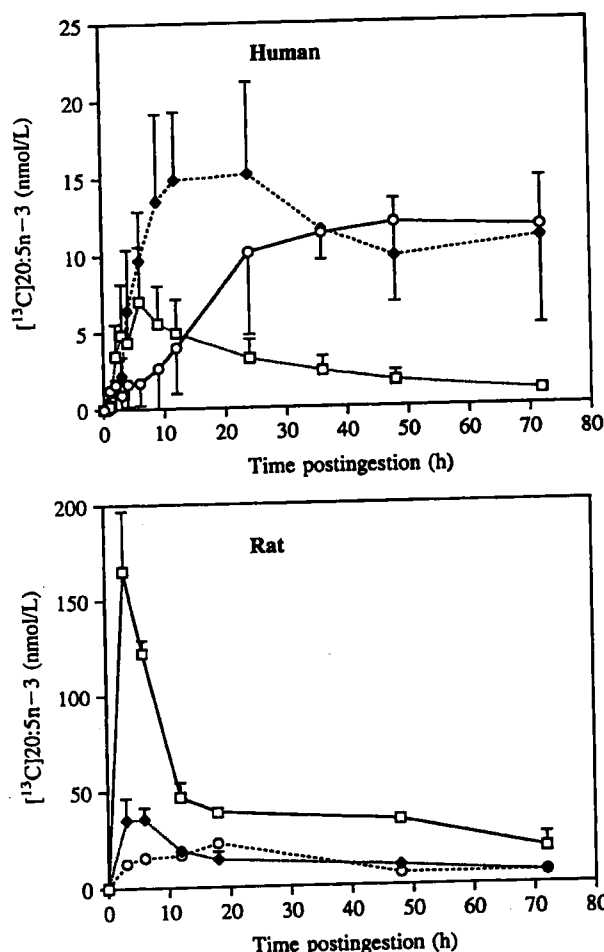


FIGURE 7. Appearance of [^{13}C]20:5n-3 in plasma triacylglycerols ($\square$), phospholipids ($\blacklozenge$), and cholesterol esters ($\circ$) in humans and rats as a function of time after ingestion of a tracer dose of [^{13}C]22:6-triacylglycerol. The amount of [^{13}C]20:5n-3 in each lipid class was calculated from $\delta^{13}\text{C}\%$ values and from endogenous 20:5n-3 concentrations. Each point represents the $\bar{x} \pm \text{SD}$ of three human subjects and the $\bar{x} \pm \text{SEM}$ of three to five rats.

22:5n-3 labeling in platelet phosphatidylcholine and phosphatidylethanolamine was comparable. In contrast, the appearance of [^{13}C]20:5n-3 was weak in red cell phosphatidylcholine as a function of time and the $\delta^{13}\text{C}\%$ values were not significantly different from baseline. No $\delta^{13}\text{C}\%$ variations were observed with [^{13}C]22:5n-3 in red blood cell phosphatidylcholine (not shown).

Selectivity of lecithin-cholesterol acyltransferase

The amounts of [^{13}C]20:5n-3 and [^{13}C]22:6n-3 esterified in HDL phosphatidylcholine and HDL-CE were calculated, as a function of time, from the ^{13}C abundance and the endogenous content of these n-3 fatty acids (Table 3). An important factor in the production of plasma CE is the enzyme lecithin-cholesterol acyltransferase (LCAT), which utilizes a fatty acyl moiety of HDL phosphatidylcholine to convert cholesterol to CE. To determine whether differences in the formation of LCAT products were due to enzyme selectivity, the ratio between the amount of individual [^{13}C]CE and [^{13}C]phosphatidylcholine

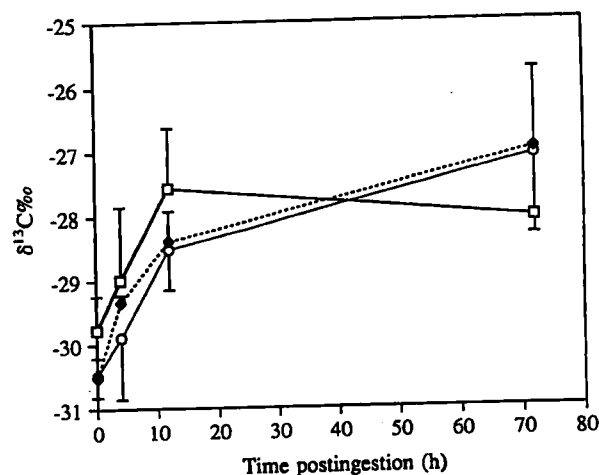


FIGURE 8. Appearance of retroconversion products in human platelet [^{13}C]22:5n-3-phosphatidylcholine ($\square$), [^{13}C]20:5n-3-phosphatidylcholine ($\blacklozenge$), and [^{13}C]22:5n-3-phosphatidylethanolamine ($\circ$) as a function of time after ingestion of a tracer dose of [^{13}C]22:6-triacylglycerol. Each point represents the $\bar{x} \pm \text{SD}$ of three human subjects.

molecular species in HDL, for the same fatty acid, was calculated. This can be considered a selectivity factor, for which a low ratio indicates a poor reactive substrate and a high ratio indicates selective utilization of a certain molecular species. LCAT selectively utilized 20:5n-3-containing phosphatidylcholine (ratio = 0.56 and 1.23 at 72 h in humans and rats, respectively) whereas 22:6n-3-containing phosphatidylcholine was poorly utilized (ratio < 1). Rat LCAT presented a higher selectivity for 20:5n-3 and 22:6n-3 than did human LCAT.

DISCUSSION

Previous studies from our group (15, 19) and others (23, 24) have shown the potential of GCC-IRMS to trace [^{13}C]fatty acids in lipid classes, in vivo. This approach has several attractive features, including the innocuity of stable isotopes for biomedical studies in humans and the very high sensitivity of detection. We took advantage of these characteristics to investigate the in vivo conversion of [^{13}C]22:6n-3 into [^{13}C]22:5n-3 and [^{13}C]20:5n-3 in humans and rats under nonsupplemented diet conditions. ^{13}C detection had to be sensitive enough to take into account tiny variations in the molecular ^{13}C content to measure the retroconversion of endogenous 22:6n-3, without dietary supplementation with this fatty acid, which may have produced a mass effect to enhance an otherwise minor back reaction. Detection as low as 0.001 atom % (< 1 $\delta^{13}\text{C}\%$) was reported previously in characterizing this method for fatty acid analysis (15).

The natural ^{13}C abundance of the three fatty acids in lipid pools differed significantly. The reasons for the higher ^{13}C abundance in 22:6n-3 are unclear. It may have resulted from differences in ^{13}C abundance in the n-3 fatty acids supplied by food or it may have been that 22:6n-3 originated from a different source than did 22:5n-3 and 20:5n-3; 22:6n-3 may have come directly from the diet (eg, from fish or marine food) whereas 20:5n-3 and 22:5n-3 may have come from the



TABLE 3

Lecithin-cholesterol acyltransferase selectivity¹

Fatty acid and time postingestion	Humans			Rats		
	HDL CE	HDL PC	CE:PC	HDL CE	HDL PC	CE:PC
	nmol/L plasma	nmol/L plasma		nmol/L plasma	nmol/L plasma	
[¹³ C]22:6n-3						
12 h	23.8 ± 3.4	831.7 ± 121.3	0.03	111.3 ± 16.5	1007.9 ± 120.1	0.11
18 or 24 h ²	43.0 ± 5.5	979.9 ± 24.7	0.04	139.6 ± 20.7	1128.0 ± 122.0	0.12
48 h	63.1 ± 10.7	909.1 ± 53.4	0.07	145.1 ± 16.2	882.6 ± 114.0	0.16
72 h	68.3 ± 11.9	802.1 ± 52.1	0.09	100.9 ± 26.5	684.1 ± 143.0	0.15
[¹³ C]20:5n-3						
12 h	1.2 ± 0.9	8.2 ± 3.0	0.15	14.9 ± 2.1	13.1 ± 2.4	1.14
18 or 24 h ²	3.4 ± 1.5	8.2 ± 0.9	0.41	19.5 ± 2.4	13.7 ± 2.1	1.42
48 h	3.4 ± 1.5	6.4 ± 0.9	0.52	7.9 ± 1.8	7.3 ± 0.9	1.08
72 h	3.0 ± 1.5	5.5 ± 1.8	0.56	4.9 ± 1.2	4.0 ± 0.3	1.23

¹ $\bar{x} \pm \text{SEM}$ of three to five rats and the $\bar{x} \pm \text{SD}$ of three human subjects.² 18 h for rats and 24 h for humans.

desaturation and elongation of 18:3n-3 of plant origin with a ¹³C abundance different from marine food. Determination of these uncertainties requires analyses of the natural ¹³C abundance in different dietary n-3 PUFAs. In the present study these observations may suggest a minimal interconversion between these n-3 PUFAs, when diet is not supplemented, because extensive desaturation and elongation and retroconversion would lead to an equilibrium of ¹³C abundance within these n-3 PUFAs.

The overall [¹³C]22:6n-3 apparent retroconversion was lower in humans than in rats, where it reached 9% of the total plasma [¹³C]22:6n-3, compared with only 1.4% in humans. This does not result from a difference between plasma 22:6n-3 endogenous concentrations, which were comparable in humans (141 μmol/L plasma) and rats (138 μmol/L plasma). It probably comes more from a different endogenous distribution of n-3 PUFAs within lipid classes. For example, 41% of the total plasma 22:6n-3 was esterified in triacylglycerols in rats, compared with only 9% in humans. The differences between the affinity and reaction rates of the enzymes of hepatic de novo synthesis or between the deacylation-reacylation pathways, in rats and humans, might generate different amounts of hepatic 22:6n-3-CoA available for retroconversion. The weak basal apparent retroconversion in humans favors the stimulation of this pathway when high amounts of dietary fatty acids are fed to humans.

Retroconversion of 22:6n-3 leads to 20:5n-3 by one cycle of β-oxidation on a 2,7,10,13,16,19-22:6-CoA intermediate generated after the disappearance of the 4-*cis* double bond without chain shortening (25, 26). This 4-*cis* double bond is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase after introduction of the 2-*trans* double bond by acyl-CoA oxidase in peroxisomes and the action of the Δ³,Δ²-enoyl-CoA isomerase. How the formation of [¹³C]22:5n-3 is linked to this metabolism is still under investigation. In vitro studies originally suggested that this PUFA would mostly originate from the chain elongation of the retroconverted 20:5n-3 (27, 28). This finding is supported by labeling values in rats, in whom [¹³C]22:5n-3 labeling was lower than [¹³C]20:5n-3 labeling; however, it is not supported by values in humans, in whom both enrichments were equivalent. It is possible that in humans the elongation of 20:5n-3 to 22:5n-3 is not rate-limiting

compared with the β-oxidation of 22:6n-3. It has also been suggested that 22:5n-3 is generated through reduction of the common intermediate of β-oxidation—2,7,10,13,16,19-22:6-CoA—by 2-enoyl-CoA reductase (H Schulz, personal communication, 1995), which has been purified and studied by several investigators (29). In this latter reaction scheme 20:5n-3 and 22:5n-3 are independent compounds and this would explain why we did not find a consistent precursor-product relation between 20:5n-3 and 22:5n-3 in vivo.

The distribution of [¹³C]22:6n-3 within plasma lipid classes after absorption of a single dose of [¹³C]22:6-triacylglycerol in humans showed the rapid appearance of this fatty acid in triacylglycerol as soon as 1 h after intake with a maximum at 2 h. In rats, the first time point studied was 3 h, to avoid an artifactual delay in absorption, which could have been observed at 1–2 h because of animal stress resulting from gastric intubation. The highest enrichment observed was at 3 h, likely placing the maximum at ≈2 h, as in humans. This favors an efficient absorption of triacylglycerols rich in 22:6n-3. Both retroconversion products—[¹³C]20:5n-3 and [¹³C]22:5n-3—also appeared rapidly in triacylglycerol, with a maximum only slightly delayed from the appearance of [¹³C]22:6n-3. These plasma n-3 PUFA-rich triacylglycerols were good substrates for lipoprotein lipase and hepatic lipase in view of their rapid disappearance from plasma. Although the liver is assumed to be the site with the most efficient retroconversion in the organism (27), the detection of retroconversion products, as early as 1 h in human plasma, might suggest that some retroconversion already took place in intestinal mucosa during the chylomicron formation.

The incorporation of [¹³C]22:6n-3 into phospholipids and CEs was more progressive, with a final accumulation in phospholipids in both humans and rats. However, the amount of [¹³C]22:6n-3 was higher in human phospholipids and this tracer was distributed within lipid classes as endogenous 22:6n-3 in both species. Tracer accumulation in rat triacylglycerols was higher than in humans and was equally distributed among phospholipids and triacylglycerols at the plateau, matching the endogenous distribution. [¹³C]22:5n-3 followed the same labeling pattern as 22:6n-3, with a final tracer accumulation in phospholipids, especially in humans. The reason for the high accumulation and long life span of [¹³C]22:



6n-3-phospholipid is not clear. It probably involves the enzyme specificity of the de novo synthesis toward diacylglycerol substrates (30) and the poor reactivity of 22:6n-3 in phospholipids with LCAT (31, 32) or hepatic lipase. Whatever the mechanisms, the high concentration of 22:6n-3-phospholipid in plasma may stimulate its utilization by tissues. Because, it has been reported that phospholipids supply PUFAs to the brain (33), it is therefore possible that 22:6n-3-phospholipids may be a carrier of 22:6n-3 to the brain, which is specifically rich in this PUFA.

In contrast, [¹³C]20:5n-3 presented a different labeling distribution and accumulation. The labeling of 20:5n-3 in phospholipids and CEs relative to triacylglycerols was more pronounced than for the two other fatty acids of the series, but the most striking difference was the accumulation of [¹³C]20:5n-3 in CEs, in both humans and rats. In plasma, LCAT transesterifies the *sn*-2 fatty acid of a phospholipid molecule to the 3-hydroxyl group of cholesterol, resulting in lyso-phosphatidylcholine and CE. The phosphatidylcholine of newly formed HDL is the best substrate for this enzyme. Because the *sn*-2 position of phosphatidylcholine is mostly occupied by a PUFA, LCAT generates saturated lyso-phosphatidylcholine and unsaturated CE. However, recent studies using purified LCAT and defined phosphatidylcholine molecular species have shown that the specificity of LCAT for the *sn*-2 position of phosphatidylcholine depends on the nature of the *sn*-2 acyl group, especially the chain length (34).

It has been shown that LCAT activity is decreased in fish oil-fed monkeys (35) and that LCAT is inhibited by phosphatidylcholine containing 22:6n-3 (31). We showed the synthesis of very low amounts of [¹³C]22:6n-3-CE, despite the high concentration of HDL phosphatidylcholine substrate in both species. The selectivity factor for 22:6n-3 utilization by LCAT is low, in contrast with this factor for 20:5n-3. The substantial formation of [¹³C]20:5n-3-CE matches the interesting *in vitro* finding from Subbaiah et al (34). Using purified LCAT and well-defined phosphatidylcholine molecular species, they showed that human LCAT is impaired in the presence of 16:0-20:4- and 16:0-22:6-phosphatidylcholine, but not in the presence of 16:0-20:5n-3-phosphatidylcholine (34, 36). This effect was related to the fatty acyl chain length, which is shorter for 20:5n-3 than for 20:4n-6 and 22:6n-3 (37). This result is also consistent with the extensive formation of 20:5n-3-CE and the weak formation of 22:6n-3-CE after feeding fish oil to humans (32).

The present study confirms previous observations *in vitro*, avoiding the possible inhibition of LCAT binding to recombined substrate particles containing only one type of synthetic phosphatidylcholine (38). Using tracer [¹³C]22:6n-3 neither modifies the overall fatty acid composition of plasma phospholipid and their species distribution, nor the size, shape, lipid composition, or fluidity of lipoprotein particles, contrary to diet-induced modifications that may indirectly influence the LCAT reactivity for its substrates (35). The selectivity factor for 20:5n-3 and 22:6n-3 was also higher in rats than in humans, indicating that rat LCAT is less inhibited by PUFAs at the *sn*-2 position than is human LCAT. This agrees with the fact that rat plasma CEs are enriched in 20:4n-6 compared with human plasma CEs, which are rich in 18:2n-6, and with *in vitro* results from Subbaiah et al reporting a higher affinity of LCAT in humans than in rats for the *sn*-1 position of

16:0-22:6n-3-phosphatidylcholine (34). The different LCAT reactivities toward 20:5n-3 and 22:6n-3 are probably related to their different antiatherogenic properties observed after fish oil feeding (4, 35). It has been reported that 22:6n-3-rich fish oil reduced the ratio of LDL to HDL to a greater degree than did the 20:5n-3-rich oil, and that only 22:6n-3-rich oil increased HDL₂ (4). Moreover, the decreased LCAT reactivity by 22:6n-3 would result in smaller LDL particles and thus would be a better antiatherogenic agent than 20:5n-3 (35).

Despite the very low concentrations of 22:6n-3 retroconversion products in plasma, we still detected some incorporation of such products into platelet phospholipids occurring rapidly after tracer intake, within the time period of maximal circulation of unesterified fatty acids bound to albumin (19). Because platelets do not have retroconversion enzymes, we conclude that both 20:5n-3 and 22:5n-3 are substrates of the Lands pathway (39), *in vivo*.

We showed that the basal apparent retroconversion of 22:6n-3 is a minor pathway in humans. However, this situation cannot be extrapolated to findings from chronic and long-term dietary supplementation with relatively large amounts of fish oil. Because retroconversion might be stimulated by high concentrations of substrate, the amount of 22:6n-3 to be used as a supplement (eg, in milk-based formulas for newborns) should be determined to prevent the synthesis of undesirable amounts of 20:5n-3. In addition, this experiment based on isotope substitution, showed that the differences between 22:6n-3 and 20:5n-3 for their transport in plasma lipid classes and their metabolism through LCAT are related to their specific properties and not to indirect effects of fish oil supplement on lipids or on the biophysical indexes of lipoprotein particles. ■

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Arachidonic acid status correlates with first year growth in preterm infants

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ABSTRACT Diets deficient in the ω -6 fatty acid linoleic acid reduce arachidonic acid (Ach) concentrations and retard growth of developing animals and humans. Nevertheless, plasma phosphatidylcholine Ach concentrations declined from 84 ± 23 mg/liter at birth to a nadir of 38 ± 11 mg/liter at 4 mo of age in preterm infants fed commercial formulas with linoleic acid, and weight normalized to that of term infants fell progressively beginning at 2 mo of age. The nadir of plasma phosphatidylcholine Ach (31 ± 7 mg/liter) and growth were further reduced by formula containing marine oil compared with the commercial formulas. Ach status (defined as the mean plasma phosphatidylcholine Ach concentration at 2, 4, and 6.5 mo) correlated with one or more measures of normalized growth through 12 mo. Ach status and maternal height accounted for as much as 59% of the weight variance and 68% of the length variance in infants fed standard formulas. Better Ach status was not from higher energy intakes. A conditional Ach deficiency in preterm infants may contribute to growth over the first year of life. On the strength of the relationship between Ach status and growth, we hypothesize that dietary Ach could improve first year growth of preterm infants.

Despite recent advances in neonatal intensive care, preterm infants do not achieve first year growth equivalent to that of infants born at term (1-5). Moreover, preterm infants remain smaller than term infants after the first year of life (6, 7), and poorer individual growth is associated with poorer mental and motor performance (8-11). That infants with chronic lung disease have poorer growth after discharge is well known (12-14); however, preterm infants free of chronic illness and fed standard formulas had progressive declines in normalized weight, weight-to-length ratio, and head circumference beginning 2 mo after expected delivery (15), as has been reported (1, 3).

Erythrocyte phospholipid arachidonic acid (Ach) in these infants also fell progressively, reaching a nadir 4 mo after expected term delivery (16) below that seen in formula-fed term infants of equivalent age (17). The nadir in plasma phospholipid Ach concentration coincided with the onset of the decline in normalized weight. The mean concentration of Ach in plasma phosphatidylcholine (PtdCho) of preterm infants at birth is 84 ± 23 mg/liter (mean $\pm$ SD, range 43-152, $n = 86$, unpublished data). When these infants were 3 weeks old, this value was 67 mg/liter (range 41-136 mg/liter, $n = 59$), and by 4 mo after term this value was 38 ± 11 mg/liter ($n = 29$). Direct evidence that normalized growth might relate to Ach status came from the observation that marine oil-supplemented formula further decreased the concentrations of plasma PtdCho Ach (31 ± 7 mg/liter) (16) and normalized weights (15) compared with standard formula. Plasma Ptd-

Cho linoleic acid (Lin) concentration remained high and was unaffected by marine oil supplementation.

Diets deficient in the essential ω 6 fatty acid, Lin, cause declines in phospholipid Ach (18) and impair growth in developing animals (19, 20) and human infants (21). Ach restored growth in ω 6-deficient rats (22) better than Lin, and diets with Ach compared with those without Ach increased tissue Ach and growth in young alligators (23). Holman and coworkers (24-27) have shown that plasma phospholipid Ach declines in a number of diseases despite apparently good Lin intakes (24-27). In this study, the growth of premature infants in the first year of life was compared to plasma PtdCho Ach concentration, which declined with both time and low intakes of marine oil ω 3 fatty acids (0.25% of energy) in infants consuming up to 16% of energy as Lin (16).

SUBJECTS AND METHODS

Selection of Patients. Preterm infants (>725 g and <1400 g) were eligible for this study if they were admitted to the Newborn Center at The University of Tennessee, Memphis. Although infants in this birth-weight range are at high risk for medical complications, those selected for this study had fewer and milder complications than the population as a whole. Infants were not eligible if they had (i) a weight for gestational age less than the fifth percentile, (ii) intraventricular hemorrhage $>$ grade 2 (28), (iii) retinopathy of prematurity $>$ stage 2 (29), (iv) a need for mechanical ventilation after achieving enteral intakes >454 kJ (>110 kcal/kg per day), (v) surgical intervention for necrotizing enterocolitis, or (vi) a maternal history of cocaine or alcohol abuse. Infants were enrolled after obtaining parental consent, according to an Institutional Review Board-approved protocol and randomly assigned to receive either commercially available formulas [Similac Special Care (PT-A) followed by Similac With Iron (T-A); Ross Laboratories, Columbus, OH] or experimental formulas identical except containing marine oil ω 3 fatty acids as 0.5% of total fatty acids (Table 1) (16). Infants were enrolled and followed until 12 mo (note that this and all ages described were calculated from the age of expected delivery).

Data from a total of 59 infants were available for analyzing the relationship between growth and essential fatty acid status. Twenty additional infants were enrolled, but data were not available because they failed to complete follow-up through 6.5 mo ($n = 13$), did not receive their assigned study formula consistently to 9 mo ($n = 5$), or were outliers (age at follow-up or biochemistry) ($n = 2$). The neonatal/perinatal characteristics of the 59 infants are shown in Table 2 by formula assignment.

Abbreviations: Ach, arachidonic acid; Lin, linoleic acid; PtdCho, phosphatidylcholine; ω 3, fatty acids of the ω -3 family derived from and including linolenic acid; ω 6, fatty acids of the ω -6 family derived from and including Lin.

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Table 1. Fatty acid composition of infant formulas

	Formula,* g/100 g of total fatty acid			
	Preterm		Term	
	PT-A	PT-B	T-A	T-B
6:0-24:0	68.0	67.1	44.4	44.0
18:1	9.9	9.8	17.1	17.0
18:2 ω 6	19.1	18.7	33.2	32.6
18:3 ω 3	3.0	3.1	4.8	4.9
20:5 ω 3		0.3		0.3
22:5 ω 3				
22:6 ω 3		0.2		0.2

Fatty acids are designated as number of carbons:number of double bonds, ω 6 or ω 3 family of fatty acids.

*Formulas contained ~48% of energy from fat. PT-A was control formula (Similac Special Care); PT-B was experimental formula. Term (T) formula (Similac With Iron) had the same ω 3 fatty acid supplementation as corresponding preterm (PT) formula.

Experimental Design. Infants were enrolled shortly after reaching intakes >454 kJ (110 kcal)/kg per day of a preterm formula. At enrollment, they were assigned a gestational age based on dates and ultrasound before assignment to formula as described earlier. If any discrepancy of >2 weeks occurred between these assessments and physical and neurological assessment of maturity at birth (30), the assessments were averaged. Infants were fed control (PT-A) or experimental (PT-B) formula until ~1.8 kg. Thereafter, a term formula with the same ω 3 fatty acid supplementation as their assigned preterm formula (T-A or T-B, Table 1) was fed ad libitum until 9 mo of age. At term, 2, 4, 6.5, 9, and 12 mo of age, dietary intake, length, weight, and head circumference were recorded, and blood was obtained by venipuncture.

Analytical Methods. Plasma was fractionated, erythrocytes were washed and stored, lipids were extracted and purified by TLC, and phospholipid fatty acids were methylated and quantitated by capillary gas/liquid chromatography as described (16, 31). Infants were weighed undressed on a calibrated infant balance. Length was measured in duplicate by use of a measuring board with infants supine (Jim's Instrument Manufacturing, Coralville, IA). Head circumference was measured with paper measuring tape.

Statistical Methods. The National Center for Health Statistics' normal values for growth by age and gender of term infants (32) were used to generate percentiles, and growth of these premature infants was normalized against these percentiles (33). The growth index generated is equivalent to a Z-score with weight, length, and head circumference expressed as $\pm$ SD from the 50th percentile. Because Z-scores can be manipulated arithmetically, values for an individual

Table 2. Characteristics of study infants

	Control (n = 29)	Marine ω -3 supplemented (n = 30)
Birth weight, g	1054 $\pm$ 193	1139 $\pm$ 164
Gestational age, wk	28.9 $\pm$ 1.7	29.7 $\pm$ 1.8
Enrollment weight, g	1268 $\pm$ 152	1337 $\pm$ 133
Enrollment age, day	25 $\pm$ 10	24 $\pm$ 10
i.v. support, day	17 $\pm$ 8	18 $\pm$ 10
Mechanical ventilation, hr*	14.8 $\pm$ 6.6	6.6 $\pm$ 7.3
Total oxygen, hr*	63.7 $\pm$ 3.8	52.0 $\pm$ 4.6
Gravida, no.	2.4 $\pm$ 1.3	2.4 $\pm$ 1.4
Maternal height, cm	163.1 $\pm$ 7.7	164.8 $\pm$ 7.9
Maternal age, yr	23 $\pm$ 6	23 $\pm$ 6
Race, Black/Caucasian	26/3	25/5

*Mean $\pm$ SD of individual logarithm of hr converted to hr (mean $\pm$ SD).

infant can be averaged across time or values within a dietary group can be averaged at a given age.

Infants were ranked into quartiles on the basis of their plasma PtdCho Ach concentration at 2 mo, and the mean plasma PtdCho Ach concentrations of each quartile were compared (mg/liter) by repeated-measures ANOVA for time (2, 4, and 6.5 mo), quartile ranking, and interaction of time and rank effects. For each quartile, mean plasma PtdCho Ach concentrations did not differ significantly over time—i.e., Ach concentrations tracked between 2 and 6.5 mo. Accordingly, the mean concentration of plasma PtdCho Ach at 2, 4, and 6.5 mo was defined as Ach status and correlated with normalized growth from 2 to 6.5 mo (Fig. 1) and at each age from 2 through 12 mo (see Table 4).

A stepwise multiple regression procedure was used to determine the independent factors that affected normalized growth at each age. Birth weight was included as a potentially influential variable in all analyses. Other potential factors were maternal height, birth order, Ach status, and marine oil supplementation as a dummy variable with zero for control and one for supplemented infants. Independent variables that met the $P < 0.05$ significance level are reported (Tables 5 and 6).

Groups of infants, ranked by quartile on the basis of Ach status during the period from 2 to 6.5 mo, were compared for birth weight and energy intake from formula at 2, 4, and 6.5 mo (Table 3). Growth (weight, length, weight-to-length ratio, and head circumference) at 2–6.5 and 9–12 mo was plotted for each quartile (Fig. 2).

RESULTS

Diet Histories/Energy Intake. Most infants received energy exclusively from formula through 4 mo. By 6.5 mo, almost all were consuming a mixed diet but with ≥ 0.72 liter of formula

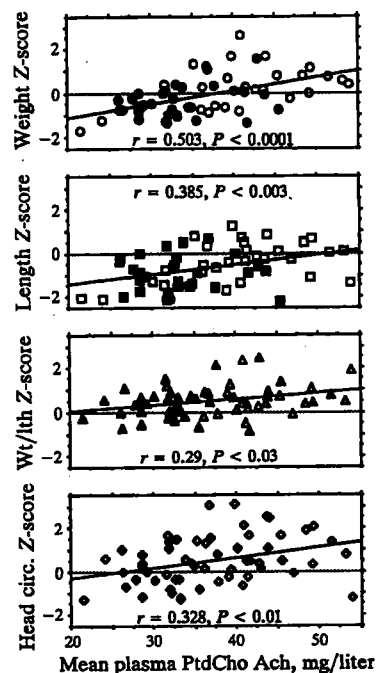


FIG. 1. Correlation of average (2, 4, and 6.5 mo) weight, length, weight-to-length (wt/lth) ratio, and head circumference (circ.) Z-scores with Ach status (average concentration of plasma PtdCho Ach at 2, 4, and 6.5 mo). Open symbols represent infants fed standard formula; filled symbols represent those supplemented with marine oil.

Table 3. Ach status by quartile: Plasma PtdCho Ach, birth weight, and reported energy intakes from formula at 2, 4, and 6.5 mo

Quartile rank (n)	Plasma PtdCho Ach, mean mg/liter (2–6.5 mo)	Birth weight, g	Reported energy intake from formula, kcal/kcal per kg		
			2 mo	4 mo	6.5 mo
Highest (15)	48.6 ± 3.9	1094 ± 158	655/136	708/118	675/92
Second (15)	39.1 ± 1.9	1052 ± 219	663/142	708/109	698/90
Third (14)	33.6 ± 1.4	1150 ± 154	572/126	663/110	691/100
Lowest (15)	28.1 ± 2.8	1098 ± 196	565/130	667/120	760/111
ANOVA by Ach quartile	P < 0.0001	NS	NS/NS	NS/NS	NS/NS

NS, not significant.

per day. After 9 mo, most received a mixed diet and cow's whole milk instead of infant formula. All quartiles for Ach status had the same birth weight and energy intake from formula (kcal or kcal/kg) at 2, 4, or 6.5 mo of age (Table 3).

Ach Status and Growth Achievement. Individual weight and length Z-scores correlated significantly ($r = 0.27$ – 0.53) with Ach status at 2, 4, 6.5, 9, and 12 mo (Table 4). Plasma PtdCho Ach accounted for 7–28% of the variance (r^2) in weight and length at these ages. The weight-to-length ratio at 2, 4, and 6.5 mo also correlated ($r = 0.25$ to 0.28) with Ach status. Head circumference was related to Ach status at 2 and 4 mo ($r = 0.34$ – 0.37) but did not relate thereafter.

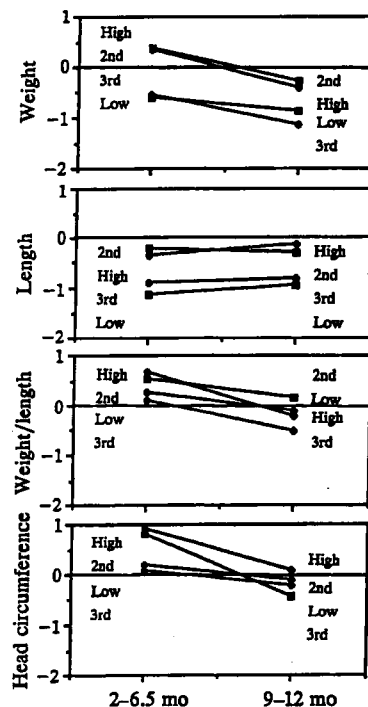


Fig. 2. Average-growth Z-scores (2–6.5 mo and 9–12 mo) of infants by quartile of Ach status (average plasma PtdCho Ach concentration from 2 to 6.5 mo). Between-groups ANOVA: (i) 2–6.5 mo—weight, $P < 0.0005$; length, $P < 0.01$; weight/length, not significant; head circumference, $P < 0.01$; (ii) 9–12 mo—weight, $P < 0.04$; length, not significant; weight/length, $P < 0.05$; head circumference, not significant. Between-quartile differences by Fisher probable least-significant difference: (i) weight Z-score: high and second vs. third and low at 2–6.5 mo and high and second vs. third at 9–12 mo, $P < 0.05$; (ii) length: high and second vs. low at 2–6.5 mo and 9–12 mo, $P < 0.05$; (iii) weight-to-length: high and second vs. third at 2–6.5 mo and second vs. third at 9–12 mo, $P < 0.05$; (iv) head circumference: high and second vs. low at 2–6.5 mo, $P < 0.05$.

Fig. 1 illustrates the relationship between Ach status and growth during the period from 2 to 6.5 mo. Each point in this figure represents the average of three measures of plasma PtdCho Ach concentration related to the average of three consecutive measures for normalized growth (Z-score). Plasma PtdCho Ach concentration accounted for 8–25% of the variance in growth during this period.

Because we knew that $\omega 3$ supplementation had decreased plasma Ach, we looked also at the relationship between Ach status and growth in infants fed only standard formulas ($n = 29$). These infants are designated by open symbols in Fig. 1. Their Ach status correlated significantly with their average normalized weight ($r = 0.43$, $P < 0.02$) and length ($r = 0.44$, $P < 0.02$) between 2 and 6.5 mo but did not correlate with their weight-to-length ratio or head circumference.

First year growth of term infants has been related to maternal height (34) and inversely related to birth order (34). These two variables, plus marine oil supplementation and Ach status, were included as independent variables in regression analyses of normalized weight (Table 5) and length (Table 6) achievement during infancy. Any potential effects of differences in birth weight were corrected by including birth weight in all models of multiple regression analyses. Some combination of Ach status, maternal height, and marine oil supplementation accounted for 21–44% of the variance in weight of the 59 study infants. Some combination of Ach status and maternal height accounted for 40–59% of the variance in weight of the 29 infants fed only commercial (control) formulas (Table 5) through 6.5 mo of age. In the

Table 4. Correlation of Ach status with normalized growth (Z-score) in the first year of life

Growth	n	Age, mo	Correlation, r	P
Weight	59	2	0.48	<0.001
	59	4	0.53	<0.001
	59	6.5	0.41	<0.001
	58	9	0.31	<0.02
	54	12	0.27	<0.05
	59	2	0.35	<0.01
Length	58	4	0.419	<0.001
	59	6.5	0.31	<0.02
	58	9	0.351	<0.01
	54	12	0.32	<0.02
	59	2	0.28	<0.03
	58	4	0.26	<0.05
Wt/length	58	6.5	0.25	<0.06
	58	9	0.33	NS
	54	12	0.13	NS
Head circ.	59	2	0.37	<0.01
	58	4	0.34	<0.01
	59	6.5	0.24	<0.07
	56	9	0.19	NS
	54	12	0.08	NS

Ages are from expected term delivery. Wt, weight; NS, not significant; circ., circumference.

Table 5. Normalized weight achievement in the first year of life

Age	Formula	Independent variable	F	P	Model r^2
Term	Both	Ach status (+)	10.66	0.002	0.21
	Control	Ach status (+)	7.78	0.01	
2 mo	Marine	Maternal height (+)	9.46	0.006	0.56
		Birth order (-)	7.34	0.02	0.23
	Both	Ach status (+)	22.41	0.0001	
		Maternal height (+)	6.52	0.01	0.35
4 mo	Control	Ach status (+)	16.73	0.0005	
		Maternal height (+)	9.44	0.006	0.59
	Marine	Birth order (-)	6.39	0.02	0.20
		Ach status (+)	12.94	0.0007	
6.5 mo	Both	Marine oil $\omega 3$ (-)	5.74	0.02	
		Maternal height (+)	5.90	0.02	0.44
	Control	Ach status (+)	13.19	0.002	
		Maternal height (+)	5.76	0.03	0.50
9 mo	Marine	Birth order (-)	10.69	0.007	0.26
		Marine oil $\omega 3$ (-)	13.92	0.0005	
12 mo	Both	Birth order (-)	4.92	0.03	0.30
		Ach status (+)	5.21	0.03	
	Control	Maternal height (+)	5.85	0.02	0.40
		Marine oil $\omega 3$ (-)	12.64	0.0008	0.20
	Both	Marine oil $\omega 3$ (-)	11.28	0.002	0.19

The stepwise regression model included birth weight (forced), maternal height, birth order, marine oil $\omega 3$ supplementation, and Ach status. Only significant variables are noted with sign (+ or -) to indicate the relationship to growth.

marine oil-supplemented infants, higher birth order was negatively related to weight in early infancy. At 9 and 12 mo, $\omega 3$ supplementation had a negative effect on weight that accounted for 20% of the variance in weight seen in the combined group of infants (Table 5).

Ach status and maternal height also accounted for 25–39% of the length variance in the 59 study infants and 58–68% of

Table 6. Normalized length achievement in the first year of life

Age	Formula	Independent variable	F	P	Model r^2
Term	Both	Ach status (+)	7.39	0.009	
		Maternal height (+)	4.59	0.04	0.25
2 mo	Control	Maternal height (+)	14.01	0.001	
		Ach status (+)	6.23	0.02	0.58
	Both	Maternal height (+)	9.26	0.004	
		Marine oil $\omega 3$ (-)	11.87	0.001	0.29
4 mo	Control	Maternal height (+)	19.24	0.0002	
		Ach status (+)	10.63	0.004	0.61
	Both	Maternal height (+)	16.18	0.0002	
		Ach status (+)	18.13	0.0001	0.39
6.5 mo	Control	Ach status (+)	19.53	0.0002	
		Maternal height (+)	25.99	0.0001	0.68
	Both	Maternal height (+)	9.88	0.003	
		Marine oil $\omega 3$ (-)	14.22	0.0004	
9 mo	Control	Birth order (-)	4.74	0.03	0.37
		Maternal height (+)	4.92	0.04	0.19
	Marine	Maternal height (+)	4.89	0.04	0.20
		Ach status (+)	11.14	0.002	
12 mo	Both	Maternal height (+)	12.07	0.001	0.29
		Ach status (+)	13.92	0.001	
	Control	Maternal height (+)	22.38	0.0001	0.61
		Ach status (+)	9.73	0.0003	0.33
	Both	Maternal height (+)	17.62	0.0001	
	Control	Maternal height (+)	11.05	0.003	0.34

The stepwise regression included birth weight (forced), maternal height, marine oil $\omega 3$ supplementation, and Ach status. Only significant variables are noted with sign (+ or -) to indicate the relationship to growth.

the variance in infants fed only commercial formulas (Table 6). At 2 and 6.5 mo of age, the negative influence of $\omega 3$ supplementation replaced the positive influence of Ach status on length in the entire group. Only maternal height was positively related to length in $\omega 3$ -supplemented infants, but that relationship was not consistent. Because $\omega 3$ supplementation significantly reduced the concentration of plasma PtdCho Ach (16), its entrance into these models as a negative influence on weight and length is consistent with a positive influence of Ach status on growth.

Infants in the two higher quartiles for Ach status achieved better normalized weight, length, and head circumference compared with the two lower quartiles (Fig. 2). The two higher quartiles had weights above the 50th percentile from 2 to 6.5 mo. Although growth in all categories, except length, declined for all quartiles of Ach status between early and late infancy, the higher quartiles compared with lower Ach quartiles continued to have higher weights and lengths at 9–12 mo (Fig. 2).

DISCUSSION

Although these preterm infants received Lin, Ach in plasma and erythrocyte phospholipids declined for months after birth (16) and remained low for ≈ 5 mo. This 5-mo period was chosen as the period of poorest individual Ach status. Large interindividual differences in both Ach status and normalized growth were seen during this 5-mo period, and Ach status and growth correlated significantly. Moreover, Ach status was one of two independent variables (the other was maternal height) that consistently accounted for a large proportion of the variance in the first-year growth achievement.

Human infants can accumulate Ach from maternal/placental transfer, consumption of human milk, and synthesis from Lin. These infants were born early in the last trimester and fed formulas without Ach; thus normal intrauterine and extrauterine accumulation of preformed Ach could not occur. Infants in this study did receive Lin well in excess of the known requirement for growth and development. This study does not address the ability of preterm infants to synthesize Ach; however, declines in Ach status after birth lead us to speculate that the rate of biosynthesis does not keep pace with the need for Ach in formula-fed, prematurely born infants. This result does not necessarily mean that these infants have poorer biosynthesis compared with term infants, older children, or adults because either an increased need for Ach or inadequate intrauterine accumulation of Ach could cause the same decline. These data do suggest that a conditional deficiency of Ach occurs despite good intakes of Lin.

After these analyses were complete, we looked at the relationship between growth and the concentrations of Lin, eicosapentaenoic acid, and docosahexaenoic acid in plasma PtdCho. Like Ach, plasma PtdCho Lin concentration (2–6.5 mo) correlated with weight and length; however, in contrast to plasma PtdCho Ach, plasma PtdCho Lin concentration was consistently high (>175 mg/liter) and did not decline with time or marine oil feeding. When Lin concentration was included with Ach status as a potential independent variable for normalized weight and length (Tables 5 and 6), Ach, but not Lin, entered the models.

Any dietary fatty acids would be expected to positively relate to growth because greater intakes of the fatty acid occur with greater energy intakes. This fact is likely the basis for the positive relationship between growth and Lin concentration, as well as our failure to find a negative relationship between growth and the concentrations of eicosapentaenoic acid and docosahexaenoic acid, even though Ach status was decreased by docosahexaenoic acid and eicosapentaenoic acid intake (16). The relationship of Ach status to growth was not confounded by intake of Ach or related to

differences in energy intake (Table 3). Only the relationship between Ach status and growth is reported here.

Differences in nutritional status may account for some variability in Ach status found at each age within infants fed the same formula. For example, the common practice of discharging preterm infants at <2 kg on formulas designed to meet the nutritional needs of much larger term infants slows nutritional recovery (35), which may reduce the ability of preterm infants to elongate/desaturate Lin to Ach (36–38). Because infants vary a great deal in plasma PtdCho Ach concentration at birth, intrauterine influences could play a role in individual Ach status. Koletzko and Braun (39) recently reported a positive relationship between plasma triglyceride Ach and body weight of premature infants at birth.

Dietary $\omega 3$ fatty acids are known to reduce phospholipid Ach (40, 41), probably by a combination of reduced Ach synthesis and simple physical replacement of phospholipid Ach by eicosapentaenoic acid and docosahexaenoic acid. Marine oil significantly decreased plasma PtdCho Ach in the half of these study infants randomized to receive supplemented formula (16). Marine oil supplementation also reduced growth (15), direct evidence that poorer Ach status was growth limiting in these infants. It is important to emphasize, however, that marine oil supplementation alone did not account for the relationship between growth and Ach status seen in these very low-birth-weight infants: Among infants fed only the commercial (control) formulas, Ach status and maternal height accounted for a large part of the variance (40–68%) in weight and length (Tables 5 and 6). Because control infants were managed according to standard practices of nutritional care, poor Ach status may relate to the routine observation that preterm infants fail to achieve normal growth in infancy. Diets containing Ach should improve Ach status (17, 42).

In conclusion, growth (15) and very long-chain $\omega 3$ and $\omega 6$ status (16) were studied in preterm infants at low risk for growth failure due to medical problems. The concentrations of plasma PtdCho Ach throughout infancy were well below those seen at birth, and erythrocyte phospholipid Ach (16) was lower than that of term infants (17). Growth equivalent to term infants was not achieved in the first year of life (15), and Ach status predicted a significant part of the variance in first year growth. Despite good intakes of the essential $\omega 6$ fatty acid, Lin, preterm infants may need dietary Ach for optimal growth.

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Understanding omega-3's

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Omega-3 fatty acids are a subset of polyunsaturated fatty acids found in marine sources as eicosapentaenoic acid and docosahexaenoic acid and in some leafy vegetables, nuts, and oils as α -linolenic acid (ALA). The metabolism of omega-3's may explain the cardioprotective effects observed in epidemiologic and experimental studies. Although most data for cardioprotective effects come from studies of marine sources, vegetable sources of omega-3 fatty acids (α -linolenic acid) may have similar effects through in vivo conversion to eicosapentaenoic acid and docosahexaenoic acid. This document will provide an overview of omega-3 fatty acids with a focus on specific sources, metabolism, safety issues, and their potential indication for cardiovascular prevention. [Am Heart J 2006;151:564-70.]

Interest in omega-3 fatty acids has grown steadily since the observation that Greenland's Eskimos have a low incidence of cardiovascular disease (CVD) in the setting of a diet rich in fatty fish.¹ Importantly, both epidemiologic and experimental data have provided evidence for a beneficial effect of omega-3 fatty acids in the prevention of CVD. In 2002, the American Heart Association released a scientific statement endorsing the use of omega-3 fatty acids in both primary and secondary prevention.²

What are omega-3's?

There are 3 types of naturally occurring fats classified by the number of double bonds present in their fatty acid side chains: saturated, monounsaturated, and polyunsaturated (Figure 1). The food industry created a fourth class, trans fats, by adding hydrogen ions to polyunsaturated fats through a process called hydrogenation (Figure 1). Polyunsaturated fats can be further classified into 2 groups based on the position of the first double bond site: omega-3 fatty acids and omega-6 fatty acids (Figure 1). The most prominent omega-6 fatty acids in the human diet are arachidonic acid (found in animal meat) and linoleic acid (found in vegetable oils, seeds, and nuts), which can be converted into arachidonic acid by a desaturase enzyme (Figure 2). Major dietary sources of omega-3's are fish containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and nuts, seeds, and vegetable

oils containing α -linolenic acid (ALA), which can be converted to EPA and then DHA by the same desaturase enzyme that converts linoleic acid to arachidonic acid (Figure 2).

The conversion of ALA to EPA is of interest because the cardioprotective effects of omega-3's have been most rigorously studied and closely associated with EPA. This conversion may explain, in part or in whole, ALA's potential benefit. Isotope-labeled ALA feeding trials have shown the conversion of ALA to EPA to vary between 0.2% and 21% and that of ALA to DHA to vary between 0% and 9%.³ Most feeding studies that measure interval changes in membrane fatty acid composition show that ALA feeding will lead to an increase in EPA but has a null effect or slight decrease in DHA levels.³ These studies, however, are somewhat limited because the conversion of ALA to EPA + DHA is likely influenced by multiple factors including, sex, competitive inhibition of desaturase by linoleic acid (Figure 2), negative feedback inhibition of desaturase by EPA + DHA (Figure 2), and timing of the sample collection.

Analysis of the Health Professional Follow-up Study cohort found that ALA's CVD protective properties were inversely related to EPA + DHA intake. The authors concluded that ALA's cardioprotective properties were contingent on conversion to EPA + DHA and that this conversion was inhibited by EPA + DHA intake⁴ (Figure 2).

Where are omega-3's?

Dietary intake of omega-3 and omega-6 fatty acids varies within and between different populations. NHANES III, the largest database of nutrient consumption of Americans, reports a median intake of EPA + DHA of 0 and <1 g/d of ALA.⁵ The ratio of omega-6 to omega-3 intake is estimated to be 20 to 1 in a modern Western diet, compared with that of our Paleolithic ancestors who ate a diet much richer in omega-3's (estimated

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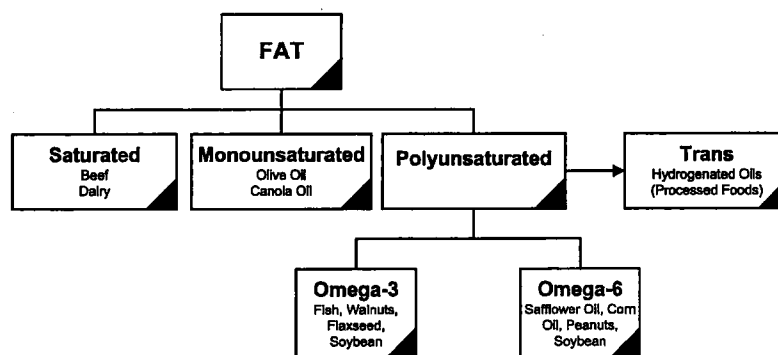
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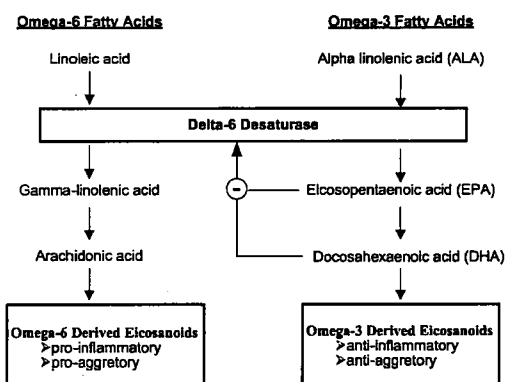
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Figure 1



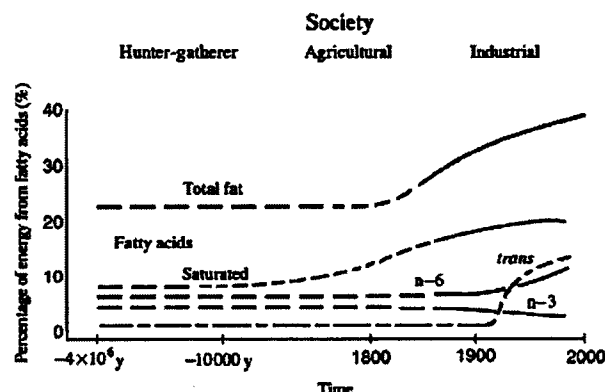
Classification of fats.

Figure 2



Metabolic pathway of omega-6 and omega-3 fatty acids.

Figure 3



Historical schematic of relative percentages of fat and intake of different fatty acids in human beings. Reproduced with permission by the American Journal of Clinical Nutrition. © Am J Clin Nutr. American Society for Clinical Nutrition.

omega-3/omega-6 ratio of 1-2:1) (Figure 3).^{6,7} This dramatic dietary shift is thought to be related to an absolute reduction in fish consumption as well as a proportionate increased consumption of domestically raised fish. Meat and fish presently contain less omega-3 and more omega-6 fatty acids than in the past, secondary to use of commercial feeds high in omega-6 and low in omega-3 content.^{8,9} Even cultivated vegetables are poor in omega-3 when compared with wild plants.¹⁰

The metabolism of omega-3's

A dietary shift toward less omega-3's and more omega-6's may significantly impact one's health because of their different metabolic pathways. Eicosanoids are a class of bioactive molecules derived from

omega-3's and omega-6's that include leukotrienes, prostaglandins, and thromboxanes (Figure 2). Eicosanoids derived from omega-6's are generally proinflammatory and proaggregatory, whereas those derived from omega-3's are predominantly anti-inflammatory and inhibit platelet aggregation.⁶ This fundamental difference may account for the cardioprotective effects of omega-3's.

Inflammation is a central component in atheroma formation and plaque rupture,¹¹ and studies have linked systemic markers of inflammation to CVD risk.¹² A cross-sectional study of 727 women in the Nurses Health Study I found dietary intake of omega-3's to be inversely related to inflammatory markers C-reactive protein, IL-6,

E-selectin, soluble intercellular cell adhesion molecule 1, and soluble vascular cell adhesion molecule 1.¹³ This relationship was consistent for ALA, EPA, and DHA independently and in aggregate.¹³ An inverse relationship between EPA + DHA intake and soluble tumor necrosis factor receptors was demonstrated in a cohort of 405 healthy men in the Health Professionals Follow-up Study and 454 healthy women in the Nurses' Health Study II.¹⁴ Levels of tumor necrosis factor receptors were lowest in subjects with a high ratio of EPA + DHA to omega-6 intake and highest in subjects with a low ratio of EPA + DHA to omega-6. In this study, ALA intake did not influence the levels of any of the inflammatory markers measured.

In addition to markers of vascular inflammation, omega-3's may beneficially influence other factors related to CVD risk: ventricular arrhythmias, thrombosis, triglycerides, apolipoprotein B, high-density lipoprotein, adhesion molecule expression in plaque, platelet-derived growth factor, nitric oxide-induced endothelial relaxation, and blood pressure.^{2,6,15-17} In general, these studies have been performed largely with EPA + DHA; few studies examine the impact of ALA on these intermediate markers of CVD. Importantly, vascular inflammation may be the common pathologic mechanism in which omega-3's impact all of these factors.

Epidemiologic data

Several important observational studies have concluded that omega-3 consumption is inversely related to CVD, especially cardiac death.¹⁸⁻²² In a study of 22071 male American physicians, those who consumed fish once a week had a 52% reduction of sudden cardiac death compared with those who ate fish less than once a month ($P = .04$).²⁰ There was no additional protective effect for men eating fish in amounts greater than once a week. Although a reduction in sudden cardiac death and total mortality was observed, there was no relationship between omega-3 consumption and myocardial infarction, nonsudden cardiac death, or CVD mortality. The Nurses Health Study of 84688 women showed an inverse relationship between consumption of EPA + DHA and both fatal and nonfatal CVD events, whereas ALA intake was only significantly related to a reduction in fatal CVD events.²¹ In the MRFIT trial of 12866 men, dietary intake of both EPA + DHA and ALA was inversely related to coronary heart disease, all CVD events, and all-cause mortality.

In the Honolulu Heart Program study of 8006 men, low fish intake was defined as consumption of fish less than twice a week and high fish intake as that ≥ 2 times a week.²³ There was no difference in the incidence of coronary heart disease between the cohorts, but this lack of association may have been secondary to a threshold

effect. Maximal reduction of sudden cardiac death in the Physicians Health Study was seen at a fish intake of once a week. A threshold effect may also explain the lack of association between fish consumption and coronary heart disease in the Health Professionals Follow-up Study.²⁴ Although a 4-fold difference in fish consumption existed between the highest and lowest quintile of participants in this study, the mean fish intake in the lowest quintile was still 1.2 servings/wk.

In addition to dietary intake studies, omega-3 fatty acid whole blood²⁵ and platelet phospholipid content¹⁶ have been shown to be inversely related to CVD death.

Prospective randomized trials

The impact of omega-3's on cardiovascular end points has been evaluated in 4 well-designed secondary prevention trials. These trials evaluated omega-3's either as part of a comprehensive diet plan or as a capsule supplement, and although these trials differed in many respects, their findings were consistent and compelling—omega-3's are effective for secondary prevention of cardiovascular events.

The DART²⁶ randomized 2033 post-myocardial infarction Welsh men to one of the following groups: (1) sensible eating (placebo); (2) low fat with an increased polyunsaturated-to-saturated ratio; (3) fatty fish consumption twice a week or 1.5 g fish oil capsules/d; (4) increased dietary fiber; or (5) a combination of the above. At 2 years, there was a 4-fold increase in EPA intake in the fish advice group (22% were taking fish oil capsules instead of eating fish).

All-cause mortality and ischemic heart disease events were not reduced in the high-fiber and low-fat groups. The group advised to consume fatty fish twice a week or take fish oil capsules had a 29% (3.5% absolute risk, $P < .05$) reduction in all-cause mortality attributable to a reduction in ischemic deaths, whereas nonfatal ischemic heart disease events were not reduced. A survey of 47% of DART participants still living 10 years after the trial's conclusion showed no long-term survival benefit in any of the intervention groups.²⁷ This lack of sustained benefit may be caused by the substantial reduction in the difference in fish and fish oil intake between the intervention and placebo groups observed during the trial.²⁷

The Indian Experiment of Infarct Survival 4 trial²⁸ randomized 360 patients with acute coronary syndrome to 1 of 3 interventions: (1) placebo; (2) fish oil capsules (1.8 g/d EPA + DHA); and (3) 20 g/d of mustard oil (2.9 g/d of ALA). After 1 year, total cardiac events (sudden cardiac death, nonfatal reinfarction, cardiac death) were reduced from 34.7% in the placebo group to 28% in the mustard oil group ($P < .01$) and to 24.5% in the fish oil group ($P < .01$). Statistically significant reductions were seen in patients in both the fish oil and the mustard oil

groups for nonfatal infarctions, angina, arrhythmias, heart failure, and left ventricular hypertrophy. Only patients in the fish oil group had a statistically significant reduction in all cardiac deaths when compared with those in the placebo group.

The Lyon Heart Study²⁹ compared a Mediterranean ALA-rich diet with a prudent diet in 608 post-myocardial infarction patients. After an average follow-up of 27 months, the Mediterranean ALA-rich diet group achieved a 70% reduction in all deaths ($P = .02$), 76% reduction in cardiac death ($P = .02$), and a 73% reduction in the primary end point of cardiac death and nonfatal myocardial infarction ($P = .001$) after multivariate adjustment. A follow-up assessment at 46 months revealed continued good compliance with the Mediterranean ALA-rich diet and similar reductions in total mortality and cardiovascular events.³⁰ Because subjects in the Mediterranean diet group made multiple dietary changes, it is not possible to determine if ALA was specifically responsible for the positive results; nonetheless, it is clear from this study that ALA can be part of a cardioprotective Mediterranean diet.

The GISSI-Prevenzione trial,³¹ an open-label design trial, randomized 11 324 post-myocardial infarction patients to 1 of 4 groups: (1) 1 g/d of fish oil supplement; (2) 300 mg/d of vitamin E supplement; (3) both; or (4) neither in addition to intensive post-myocardial infarction care and good adherence to a Mediterranean diet with >70% of the participants eating fish at least once a week. This care did not differ between the intervention and control groups and data were collected on 99.9% of participants during the 3.5 years of intervention. The fish oil supplement group had a 15% reduction in the combined end point of death, nonfatal myocardial infarction, and nonfatal stroke ($P = .023$) when compared with the control group (no supplement). Analysis of individual end points showed a 20% reduction in death ($P < .05$), 30% reduction in cardiovascular deaths ($P = .02$), and a 45% reduction in sudden death ($P = .01$). There was no reduction in nonfatal cardiovascular events in the fish oil group. Vitamin E did not impact CVD outcomes in the presence or absence of fish oil.

All 4 trials report a reduction in secondary cardiac events with either 1.0 to 1.8 g/d of fish oil capsules/1 serving of fish per day (DART, the Indian Experiment of Infarct Survival 4 trial, GISSI) or ALA supplementation (Indian Experiment of Infarct Survival 4 trial, Lyon Heart Study). Results are similar with fish/fish oil supplemented as part of a comprehensive dietary intervention and as a single intervention (DART), fish oil capsules alone (GISSI), and ALA as a single intervention (Indian Experiment of Infarct Survival 4 trial) or as part of a comprehensive diet plan (Lyon). Of the 4 trials, GISSI provides the most convincing data because of its large size (11 324), randomized intervention with a controlled

dose (1 g/d) of fish oil, and 99.9% follow up rate, although it can be criticized for its open-label design.

Clinical use

In addition to being useful in the secondary prevention of CVD events, a prescription formulation of omega-3 fatty acids, Omacor, has gained Food and Drug Administration (FDA) approval as an adjunct to diet to reduce very high (>500 mg/dL) triglycerides in adults. Two studies of 4 g/d of Omacor demonstrated a 50% reduction in triglycerides and a 44.5% increase in low-density lipoprotein (LDL) cholesterol in 84 adults with primary hypertriglyceridemia.³² A review of 10 trials in 606 subjects with primary hypertriglyceridemia (triglycerides >150 mg/dL and/or total cholesterol >200 mg/dL) supplemented with 3.4 to 4 g/d of fish oil for 4 to 16 weeks was notable for a 16% to 45% reduction in triglycerides in all but one study.³³ Total cholesterol decreased 0% to 9.3%, high-density lipoprotein increased 0% to 13%, and changes in LDL ranged from -11.3% to +32%. Although fish oil may increase total LDL (at doses >1 g/d), the resultant LDL population may have an increased particle size that is potentially less atherogenic than small, dense LDL.³⁴ In normotriglyceridemic diabetics, fish oil supplementation modestly lowers triglycerides without any clinically significant effect on glycemic control. In hypertriglyceridemic diabetics, fish oil supplementation reduces triglycerides; however, it may increase LDL.³⁵ In three small studies ($n = 41$, $n = 48$, $n = 55$), fish oil supplementation has been used as a safe adjunct to statin therapy to further reduce triglycerides, while maintaining a reduction in LDL.³⁶⁻³⁸ The impact of ALA on triglycerides remains uncertain given that a single study of 30 adults supplemented with 4.5 g/d of ALA demonstrated no significant change in a baseline triglyceride level of 147 mg/dL.³⁹

Fish oil supplementation at 1 to 2 g/d titratable to 4 g/d in single or divided doses can be considered for patients with modest hypertriglyceridemia (200-499 mg/dL) who do not have a reversible secondary cause of hypertriglyceridemia and have failed dietary intervention. Periodic monitoring of LDL and triglycerides levels is prudent until a safe, steady, therapeutic dose is achieved. Treatment recommendations for severe hypertriglyceridemia (>500 mg/dL) are beyond the scope of this article.

Safety

The US Department of Health and Human Services Agency for Healthcare Research and Quality identified 148 omega-3 fatty acid studies that reported on adverse events in >20 000 subjects.⁵ In summary, gastrointestinal complaints were reported in 6.6% of the subjects

Table I. Mercury level in 5 popular fish oil supplements

Supplement brand name	Mercury level ($\mu\text{g/L}$)
CVS	10
Kirkland	<6
Nordic Ultimate	<6
Omega Brite	12
Sundown	<6

Data from Foran et al.⁴⁴**Table II.** Omega-3 fatty acid content of select food

Food item	EPA	DHA	ALA
Fish			
Catfish	Trace	0.2	0.1
Cod	Trace	0.1	Trace
Mackerel	0.9	1.4	0.2
Salmon			
Farmed	0.6	1.3	Trace
Wild	0.3	1.1	0.3
Canned	0.9	0.8	Trace
Salmon, Chinook	1.0	0.9	Trace
Swordfish	0.1	0.5	0.2
Tuna, Bluefin	0.3	0.9	—
Tuna, Light			
Canned in oil	Trace	0.1	Trace
Canned in water	Trace	0.2	Trace
Tuna, White			
Canned in oil	Trace	0.2	0.2
Canned in water	0.2	0.6	Trace
Shellfish			
Lobster	—	—	—
Mussels	0.2	0.3	Trace
Shrimp	0.3	0.2	Trace
Nuts and seeds			
Butternuts	—	—	8.7
Flaxseed	—	—	18.1
Walnuts	—	—	9.1
Plant oils			
Canola	—	—	9.3
Flaxseed	—	—	53.3

Measurements are expressed in grams per 100 grams (3.5 oz) of food item.
Trace = <0.1; (—) = 0 or no data.Adapted from the US Department of Health and Human Services.⁵

taking omega-3's versus 4.3% in the placebo groups. An increased incidence of bleeding was not observed, and only 1 of the 148 studies reviewed reported such an association in patients randomized to 6 g/d of omega-3. There are no reported deaths or life-threatening illness as a consequence of omega-3 consumption, and 77 of the studies reported no adverse events at all. The agency concluded that adverse events related to consumption of fish oil or ALA supplements appear to be minor. In addition, the FDA has ruled that up to 3 g/d of EPA + DHA is safe,⁴⁰ although most data are limited to <6 months. Importantly, caution should be exercised when applying safety data

Table III. Summary of American Heart Association recommendations for omega-3 fatty acid intake

Population	Recommendation
Patients without documented CHD	Eat a variety of (preferably fatty) fish at least twice a week. Include food and oils rich in ALA in your diet.
Patients with documented CHD	Consume approximately 1 g of EPA + DHA (3 g of fish oil) everyday, preferably from fatty fish. EPA + DHA (fish oil) supplements could be considered in consultation with a physician.

Data from Kris-Etherton et al.²

generated in a clinical research setting to patients in the general population.

Concerns have been raised regarding potential mercury exposure from fish and fish oil consumption. However, there have been no cases of mercury poisoning related to fish consumption reported in the United States over the last 35 years. Subclinical neural damage from chronic exposure to low levels of mercury found in fish is controversial.⁴¹ One observational study reported subtle neuropsychological changes in children who had been exposed to high levels of mercury through frequent maternal consumption of whales (1.6 μg methyl-mercury/g) during pregnancy.^{41,42} A similar study found no adverse effects in children whose mothers consumed an average of 12 servings of fish per week that contained average levels of methyl mercury (0.3 $\mu\text{g/g}$).^{41,42}

Although the risk posed by mercury exposure through fish consumption is speculative, the FDA recommends limiting consumption of fish that are high in mercury (>1 ppm or approximately 1 $\mu\text{g/g}$) to one serving (7 oz) per week.⁴³ Because the fetal brain is more susceptible than the adult brain to mercury-induced damage, the FDA and the Environmental Protection Agency recommend that pregnant women, nursing mothers, and young children avoid eating fish with high mercury content. The FDA maintains a web page (<http://www.cfsan.fda.gov>) that currently lists shark, swordfish, king mackerel, and tilefish as having a high mercury content. With the exception of Omacor, available by prescription, the FDA does not regulate fish oil supplements, and, at the time of this review, we were able to identify only one peer-reviewed English language study that had evaluated fish oil supplements for mercury content. This study examined 5 popular fish oil supplements: 3 were found to have undetectable levels of mercury (<6 $\mu\text{g/L}$) and 2 had levels between 10 and 12 $\mu\text{g/L}$ of mercury by cold vapor atomic absorption spectroscopy (Table I).⁴⁴ This level of mercury is considered negligible.

Several cohort and case-control studies evaluating the relationship between omega-3's and prostate cancer have been conducted with varying results. A meta-analysis combining the results of 4 cohort and 5 case-control studies concluded that high intake of ALA is associated with an increased risk of prostate cancer (relative risk 1.70, 95% CI 1.12-2.58).⁴⁵ The authors note that the studies were quite heterogeneous, with only 1 of the 4 prospective cohort trials and 3 of the 5 case-control studies showing a statistically significant positive association to prostate cancer. To date, null and inverse associations between EPA + DHA and prostate cancer have been reported.^{45,46}

Conclusion

Omega-3's are a unique group of polyunsaturated fats that can be found most abundantly in fatty fish, flaxseed, walnuts, soy, and canola oil (Table II). The metabolism of omega-3's from fish (EPA + DHA) and vegetables (ALA) results in the production of the same eicosanoids (thromboxane, leukotrienes, prostaglandins); however, it is unclear as to what extent ALA is metabolized into these eicosanoids and if this metabolism is directly related to its effect on CVD. It does seem clear from the 4 prospective randomized trials outlined above that both fish and plant sources of omega-3's can favorably impact cardiovascular health. The impact of omega-3's is most consistently related to the use of fish oil. Although data on reduction in cardiovascular events with plant sources of omega-3's (ALA) exist, the number of subjects studied in the Indian Experiment of Infarct Survival 4 was small and ALA supplementation was part of multiple dietary interventions in the Lyon Heart Study. Clearly, a prospective randomized controlled trial, similar to GISSI, with ALA would be very valuable. Strong cohort trial data exist for the primary prevention of cardiovascular events with omega-3's (EPA, DHA, ALA), but no prospective randomized controlled trial has adequately evaluated the effect of omega-3 fatty acids on the primary prevention of CVD. To date, no serious adverse effects of omega-3's have been identified despite trial data on 20000 subjects. Potential harm from mercury exposure can be avoided with prudent fish and fish oil supplement selection. Data on the association between ALA and prostate cancer are inconsistent and limited to case-control and cohort trials. Given this concern, if omega-3 supplementation is going to be implemented in men, fish oil may be a more prudent choice than ALA.

It is recommended that patients with known CVD consume one serving (200-400 g) of fatty fish (Table II) or 1 g/d of fish oil supplement and maintain a healthy diet that is rich in ALA (Table II). Patients with a CVD risk equivalent (diabetes, peripheral vascular disease, etc) should consider consumption of a single serving of

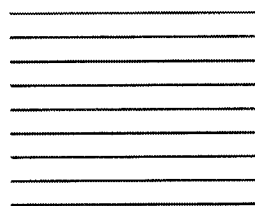
fatty fish or 1 g/d of fish oil supplement and eat a healthy diet rich in ALA. Fish oil supplements may be particularly helpful in patients with known CVD or CVD risk equivalents and hypertriglyceridemia. For patients without known CVD, a single serving of fatty fish approximately once or twice a week and a diet rich in ALA should be encouraged. It is prudent to avoid fish that contain high levels of mercury as defined by the FDA. These recommendations are in agreement with the American Heart Association's scientific statement (Table III).

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DRI



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The serpent has been a symbol of long life, healing, and knowledge among almost all cultures and religions since the beginning of recorded history. The serpent adopted as a logo-type by the Institute of Medicine is a relief carving from ancient Greece, now held by the Staatliche Museen in Berlin.

8

Dietary Fats: Total Fat and Fatty Acids

SUMMARY

Fat is a major source of fuel energy for the body and aids in the absorption of fat-soluble vitamins and carotenoids. Neither an Adequate Intake (AI) nor Recommended Dietary Allowance (RDA) is set for total fat because there are insufficient data to determine a defined level of fat intake at which risk of inadequacy or prevention of chronic disease occurs. An Acceptable Macronutrient Distribution Range (AMDR), however, has been estimated for total fat—it is 20 to 35 percent of energy (see Chapter 11). A Tolerable Upper Intake Level (UL) is not set for total fat because there is no defined intake level of fat at which an adverse effect occurs.

Saturated fatty acids are synthesized by the body to provide an adequate level needed for their physiological and structural functions; they have no known role in preventing chronic diseases. Therefore, neither an AI nor RDA is set for saturated fatty acids. There is a positive linear trend between total saturated fatty acid intake and total and low density lipoprotein (LDL) cholesterol concentration and increased risk of coronary heart disease (CHD). A UL is not set for saturated fatty acids because any incremental increase in saturated fatty acid intake increases CHD risk. It is neither possible nor advisable to achieve 0 percent of energy from saturated fatty acids in typical whole-food diets. This is because all fat and oil sources are mixtures of fatty acids, and consuming 0 percent of energy would require extraordinary changes in patterns of dietary intake. Such extraordinary adjustments may introduce undesirable effects (e.g., inadequate intakes of protein and

certain micronutrients) and unknown and unquantifiable health risks. The AMDR for total fat is set at 20 to 35 percent of energy. It is possible to have a diet low in saturated fatty acids by following the dietary guidance provided in Chapter 11.

n-9 *cis* Monounsaturated fatty acids are synthesized by the body and have no known independent beneficial role in human health and are not required in the diet. Therefore, neither an AI nor an RDA is set. There is insufficient evidence to set a UL for *n*-9 *cis* monounsaturated fatty acids.

Linoleic acid is the only *n*-6 polyunsaturated fatty acid that is an essential fatty acid; it serves as a precursor to eicosanoids. A lack of dietary *n*-6 polyunsaturated fatty acids is characterized by rough and scaly skin, dermatitis, and an elevated eicosatrienoic acid:arachidonic acid (triene:tetraene) ratio. The AI for linoleic acid is based on the median intake in the United States where an *n*-6 fatty acid deficiency is nonexistent in healthy individuals. The AI is 17 g/d for young men and 12 g/d for young women. While intake levels much lower than the AI occur in the United States without the presence of a deficiency, the AI can provide the beneficial health effects associated with the consumption of linoleic acid (see Chapter 11). There is insufficient evidence to set a UL for *n*-6 polyunsaturated fatty acids.

n-3 Polyunsaturated fatty acids play an important role as structural membrane lipids, particularly in nerve tissue and the retina, and are precursors to eicosanoids. A lack of α -linolenic acid in the diet can result in clinical symptoms of a deficiency (e.g., scaly dermatitis). An AI is set for α -linolenic acid based on median intakes in the United States where an *n*-3 fatty acid deficiency is nonexistent in healthy individuals. The AI is 1.6 and 1.1 g/d for men and women, respectively. While intake levels much lower than the AI occur in the United States without the presence of a deficiency, the AI can provide the beneficial health effects associated with the consumption of *n*-3 fatty acids (see Chapter 11). There is insufficient evidence to set a UL for *n*-3 fatty acids.

Trans fatty acids are not essential and provide no known benefit to human health. Therefore, no AI or RDA is set. As with saturated fatty acids, there is a positive linear trend between *trans* fatty acid intake and LDL cholesterol concentration, and therefore increased risk of CHD. A UL is not set for *trans* fatty acids because any incremental increase in *trans* fatty acid intake increases CHD risk. Because *trans* fatty acids are unavoidable in ordinary, nonvegan diets, consuming 0 percent of energy would require significant changes in patterns of dietary intake. As with saturated fatty acids, such adjustments may introduce undesirable effects (e.g., elimina-

tion of commercially prepared foods, dairy products, and meats that contain *trans* fatty acids may result in inadequate intakes of protein and certain micronutrients) and unknown and unquantifiable health risks. Nevertheless, it is recommended that *trans* fatty acid consumption be as low as possible while consuming a nutritionally adequate diet. Dietary guidance in minimizing *trans* fatty acid intake is provided in Chapter 11.

BACKGROUND INFORMATION

Total Fat

Fat is a major source of fuel energy for the body. It also aids in the absorption of the fat-soluble vitamins A, D, E, and K and carotenoids. Dietary fat consists primarily (98 percent) of triacylglycerol, which is composed of one glycerol molecule esterified with three fatty acid molecules, and smaller amounts of phospholipids and sterols. Fatty acids are hydrocarbon chains that contain a methyl (CH_3 -) and a carboxyl ($-\text{COOH}$) end. The fatty acids vary in carbon chain length and degree of unsaturation (number of double bonds in the carbon chain). The fatty acids can be classified into the following categories:

- Saturated fatty acids
- *Cis* monounsaturated fatty acids
- *Cis* polyunsaturated fatty acids
 - *n*-6 fatty acids
 - *n*-3 fatty acids
- *Trans* fatty acids

Dietary fat derives from both animal and plant products. In general, animal fats have higher melting points and are solid at room temperature, which is a reflection of their high content of saturated fatty acids. Plant fats (oils) tend to have lower melting points and are liquid at room temperature (oils); this is explained by their high content of unsaturated fatty acids. Exceptions to this rule are the seed oils (e.g., coconut oil and palm kernel oil), which are high in saturated fat and solid at room temperature. *Trans* fatty acids have physical properties generally resembling saturated fatty acids and their presence tends to harden fats. In the discussion below, total fat intake refers to the intake of all forms of triacylglycerol, regardless of fatty acid composition, in terms of percentage of total energy intake.

In addition to the functions of fat and fatty acids described above, fatty acids also function in cell signaling and alter expression of specific genes

involved in lipid and carbohydrate metabolism (Jump and Clarke, 1999; Sessler and Ntambi, 1998). Fatty acids may themselves be ligands for, or serve as precursors for, the synthesis of unknown endogenous ligands for nuclear peroxisome proliferator activating receptors (Kliwer et al., 1997; Latruffe and Vamecq, 1997). These receptors are important regulators of adipogenesis, inflammation, insulin action, and neurological function.

Phospholipids

Phospholipids are a form of fat that contains one glycerol molecule that is esterified with two fatty acids and either inositol, choline, serine, or ethanolamine. Phospholipids are primarily located in the membranes of cells in the body and the globule membranes in milk. A very small amount of dietary fat occurs as phospholipid. The metabolism of phospholipids is described below for total fat. The various fatty acids that are contained in phospholipids are the same as those present in triglycerides.

Saturated Fatty Acids

The majority of dietary saturated fatty acids come from animal products such as meat and dairy products (USDA, 1996). The remaining comes from plant sources. These sources provide a series of saturated fatty acids for which the major dietary fatty acids range in chain length from 8 to 18 carbon atoms. These are:

- 8:0 Caprylic acid
- 10:0 Caproic acid
- 12:0 Lauric acid
- 14:0 Myristic acid
- 16:0 Palmitic acid
- 18:0 Stearic acid

The saturated fatty acids are not only a source of body fuel, but are also structural components of cell membranes. Various saturated fatty acids are also associated with proteins and are necessary for their normal function. Saturated fatty acids can be synthesized by the body.

Fats in general, including saturated fatty acids, play a role in providing desirable texture and palatability to foods used in the diet. Palmitic acid is particularly useful for enhancing the organoleptic properties of fats used in commercial products. Stearic acid, in contrast, has physical properties that limit the amount that can be incorporated into dietary fat.

Cis Monounsaturated Fatty Acids

Cis monounsaturated fatty acids are characterized by having one double bond with the hydrogen atoms present on the same side of the double bond. Typically, plant sources rich in *cis* monounsaturated fatty acids (e.g., canola oil, olive oil, and the high oleic safflower and sunflower oils) are liquid at room temperature. Monounsaturated fatty acids are present in foods with a double bond located at 7 (*n*-7) or 9 (*n*-9) carbon atoms from the methyl end. Monounsaturated fatty acids that are present in the diet include:

- 18:1 *n*-9 Oleic acid
- 14:1 *n*-7 Myristoleic acid
- 16:1 *n*-7 Palmitoleic acid
- 18:1 *n*-7 Vaccenic acid
- 20:1 *n*-9 Eicosenoic acid
- 22:1 *n*-9 Erucic acid

Oleic acid accounts for about 92 percent of dietary monounsaturated fatty acids. Monounsaturated fatty acids, including oleic acid and nervonic acid (24:1 *n*-9), are important in membrane structural lipids, particularly nervous tissue myelin. Other monounsaturated fatty acids, such as palmitoleic acid, are present in minor amounts in the diet.

n-6 Polyunsaturated Fatty Acids

The primary *n*-6 polyunsaturated fatty acids are:

- 18:2 Linoleic acid
- 18:3 γ -Linolenic acid
- 20:3 Dihomo- γ -linolenic acid
- 20:4 Arachidonic acid
- 22:4 Adrenic acid
- 22:5 Docosapentaenoic acid

Linoleic acid cannot be synthesized by humans and a lack of it results in adverse clinical symptoms, including a scaly rash and reduced growth. Therefore, linoleic acid is essential in the diet. Linoleic acid is the precursor to arachidonic acid, which is the substrate for eicosanoid production in tissues, is a component of membrane structural lipids, and is also important in cell signaling pathways. Dihomo- γ -linolenic acid, also formed from linoleic acid, is also an eicosanoid precursor. *n*-6 Polyunsaturated fatty acids also play critical roles in normal epithelial cell function (Jones and

Kubow, 1999). Arachidonic acid and other unsaturated fatty acids are involved with regulation of gene expression resulting in decreased expression of proteins that regulate the enzymes involved with fatty acid synthesis (Ou et al., 2001). This may partly explain the ability of unsaturated fatty acids to influence the hepatic synthesis of fatty acids.

n-3 Polyunsaturated Fatty Acids

n-3 Polyunsaturated fatty acids tend to be highly unsaturated with one of the double bonds located at 3 carbon atoms from the methyl end. This group includes:

- 18:3 α -Linolenic acid
- 20:5 Eicosapentaenoic acid
- 22:5 Docosapentaenoic acid
- 22:6 Docosahexaenoic acid

α -Linolenic acid is not synthesized by humans and a lack of it results in adverse clinical symptoms, including neurological abnormalities and poor growth. Therefore, α -linolenic acid is essential in the diet. It is the precursor for synthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are formed in varying amounts in animal tissues, especially fatty fish, but not in plant cells. EPA is the precursor of *n-3* eicosanoids, which have been shown to have beneficial effects in preventing coronary heart disease, arrhythmias, and thrombosis (Kinsella et al., 1990).

Trans Fatty Acids

Trans fatty acids are unsaturated fatty acids that contain at least one double bond in the *trans* configuration. The *trans* double-bond configuration results in a larger bond angle than the *cis* configuration, which in turn results in a more extended fatty acid carbon chain more similar to that of saturated fatty acids rather than that of *cis* unsaturated, double-bond-containing fatty acids. The conformation of the double bond impacts on the physical properties of the fatty acid. Those fatty acids containing a *trans* double bond have the potential for closer packing or aligning of acyl chains, resulting in decreased mobility; hence fluidity is reduced when compared to fatty acids containing a *cis* double bond. Partial hydrogenation of polyunsaturated oils causes isomerization of some of the remaining double bonds and migration of others, resulting in an increase in the *trans* fatty acid content and the hardening of fat. Hydrogenation of oils, such as corn oil, can result in both *cis* and *trans* double bonds anywhere between carbon 4 and carbon 16. A major *trans* fatty acid is elaidic acid (9-*trans* 18:1).

During hydrogenation of polyunsaturated fatty acids, small amounts of several other *trans* fatty acids (9-*trans*,12-*cis* 18:2; 9-*cis*,12-*trans* 18:2) are produced. In addition to these isomers, dairy fat and meats contain 9-*trans* 16:1 and conjugated dienes (9-*cis*,11-*trans* 18:2). The *trans* fatty acid content in foods tends to be higher in foods containing hydrogenated oils (Emken, 1995).

Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) is a collective term for a group of geometric and positional isomers of linoleic acid in which the *trans/cis* double bonds are conjugated; that is, the double bonds occur without an intervening carbon atom not part of a double bond. At least nine different isomers of CLA have been reported as minor constituents of food (Ha et al., 1989), but only two of the isomers, *cis*-9,*trans*-11 and *trans*-10,*cis*-12, possess biological activity (Pariza et al., 2001). There is limited evidence to suggest that the *trans*-10,*cis*-12 isomer reduces the uptake of lipids by the adipocyte, and that the *cis*-9,*trans*-11 isomer is active in inhibiting carcinogenesis. Similarly, there are limited data to show that *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers inhibit atherogenesis (Kritchevsky et al., 2000).

CLA is naturally present in dairy products and ruminant meats as a consequence of biohydrogenation in the rumen. *Butyrivibrio fibrisolvens*, a ruminant microorganism, is responsible for the production of the *cis*-9,*trans*-11 CLA isomer that is synthesized as a result of the biohydrogenation of linoleic acid (Noble et al., 1974). The *cis*-9,*trans*-11 CLA isomer may be directly absorbed or further metabolized to *trans*-11 octadecenoic acid (vaccenic acid) (Pariza et al., 2001). After absorption, vaccenic acid can then be converted back to *cis*-9,*trans*-11 CLA within mammalian cells by $\Delta 9$ desaturase (Adlof et al., 2000; Chin et al., 1994; Griinari et al., 2000; Santora et al., 2000). Additionally, the biohydrogenation of several other polyunsaturated fatty acids has been shown to produce vaccenic acid as an intermediate (Griinari and Bauman, 1999), thus providing additional substrate for the endogenous production of *cis*-9,*trans*-11 CLA. Griinari and coworkers (2000) estimate that approximately 64 percent of the CLA in cow's milk is of endogenous origin.

Verhulst and coworkers (1987) isolated a microorganism, *Propionibacterium acnes*, that appears to have the ability to convert linoleic acid to *trans*-10,*cis*-12 CLA, an isomer of CLA that is found in rumen digesta (Fellner et al., 1999). *Trans*-10 octadecenoic acid is formed in the rumen via biohydrogenation of *trans*-10,*cis*-12 CLA, and both have been reported to be found in cow's milk (Griinari and Bauman, 1999). However, endogenous production of *trans*-10,*cis*-12 CLA from *trans*-10 octadecenoic acid does not occur because mammalian cells do not possess the $\Delta 12$ desaturase enzyme (Adlof et al., 2000; Pariza et al., 2001). Therefore, any *trans*-10,*cis*-12 CLA

isomer that is reported in mammalian tissue or sera would likely originate from gastrointestinal absorption.

Physiology of Absorption, Metabolism, and Excretion

Total Fat

Absorption. Dietary fat undergoes lipolysis by lipases in the gastrointestinal tract prior to absorption. Although there are lipases in the saliva and gastric secretion, most lipolysis occurs in the small intestine. The hydrolysis of triacylglycerol is achieved through the action of pancreatic lipase, which requires colipase, also secreted by the pancreas, for activity. In the intestine, fat is emulsified with bile salts and phospholipids secreted into the intestine in bile, hydrolyzed by pancreatic enzymes, and almost completely absorbed. Pancreatic lipase has high specificity for the *sn*-1 and *sn*-3 positions of dietary triacylglycerols, resulting in the release of free fatty acids from the *sn*-1 and *sn*-3 positions and 2-monoacylglycerol. These products of digestion are absorbed into the enterocyte, and the triacylglycerols are reassembled, largely via the 2-monoacylglycerol pathway. This pathway conserves the fatty acid at the *sn*-2 position. The triacylglycerols are then assembled together with cholesterol, phospholipid, and apoproteins into chylomicrons. Following absorption, fatty acids of carbon chain length 12 or less may be transported as unesterified fatty acids bound to albumin directly to the liver via the portal vein, rather than acylated into triacylglycerols.

Dietary phospholipids are hydrolyzed by pancreatic phospholipase A₂ and cholesterol esters by pancreatic cholesterol ester hydrolase. The lysophospholipids are re-esterified and packaged together with cholesterol and triacylglycerols in intestinal lipoproteins or transported as lysophospholipid via the portal system to the liver.

Chylomicrons enter the circulation through the thoracic duct. These particles enter the circulation and within the capillaries of muscle and adipose tissue. Chylomicrons come into contact with the enzyme lipoprotein lipase, which is located on the surface of capillaries. Activation of lipoprotein lipase apolipoprotein CII, an apoprotein present on chylomicrons, results in the hydrolysis of the chylomicron triacylglycerol fatty acids. Most of the fatty acids released in this process are taken up by adipose tissue and re-esterified into triacylglycerol for storage. Triacylglycerol fatty acids also are taken up by muscle and oxidized for energy or are released into the systemic circulation and returned to the liver.

Metabolism. Most newly absorbed fatty acids enter adipose tissue for storage as triacylglycerol. However, in the postabsorptive state or during exercise when fat is needed for fuel, adipose tissue triacylglycerol undergoes lipolysis and free fatty acids are released into the circulation. Hydrolysis occurs via the action of the adipose tissue enzyme hormone-sensitive lipase. The activity of this lipase is suppressed by insulin. When plasma insulin concentrations fall in the postabsorptive state, hormone-sensitive lipase is activated to release more free fatty acids into the circulation. Thus, in the postabsorptive state, free fatty acid concentrations in plasma are high; conversely, in the postprandial state, hormone-sensitive lipase activity is suppressed and free fatty acid concentrations in plasma are low.

Free fatty acids circulate in the blood bound to albumin. The major site of fatty acid oxidation is skeletal muscle. When free fatty acid concentrations are relatively high, muscle uptake of fatty acids is also high. As in liver, fatty acids in the muscle are transported via a carnitine-dependent pathway into mitochondria where they undergo β -oxidation, which involves removal of two carbon fragments. These two carbon units enter the citric acid cycle as acetyl coenzyme A (CoA), through which they are completely oxidized to carbon dioxide with the generation of large quantities of high-energy phosphate bonds, or they condense to form ketone bodies. Muscle can oxidize both fatty acids and glucose for energy. However, the uptake of fatty acids in excess of the needs for oxidation for energy by muscle does result in temporary storage as triacylglycerol (Bessesen et al., 1995). High uptake of fatty acids by skeletal muscle also reduces glucose uptake by muscle and glucose oxidation (Pan et al., 1997; Roden et al., 1996).

Fatty acids released from adipose tissue or to a lesser extent during hydrolysis of chylomicron and very low density lipoprotein (VLDL) triacylglycerols are also taken up and oxidized by the liver. Oxidation of fatty acids containing up to 18 carbon atoms occurs mainly in the mitochondria. Oxidation of excess fatty acids in the liver, which occurs in prolonged fasting and with high intakes of medium-chain fatty acids, results in formation of large amounts of acetyl CoA that exceed the capacity for entry to the citric acid cycle. These 2-carbon acetyl CoA units condense to form ketone bodies (e.g., acetoacetate and β -hydroxybutyrate) that are released into the circulation. During starvation or prolonged low carbohydrate intake, ketone bodies can become an important alternate energy substrate to glucose for the brain and muscle. High dietary intakes of medium-chain fatty acids also result in the generation of ketone bodies. This is explained by the carnitine-independent influx of medium-chain fatty acids into the mitochondria, thus by-passing this regulatory step of fatty acid entry into β -oxidation. Fatty acids of greater than 18 carbon atoms require chain shortening in peroxisomes prior to mitochondrial β -oxidation.

Fatty acids that do not enter into oxidative pathways can be re-esterified into triacylglycerols or other lipids. The major pathway for triacylglycerol synthesis in liver is the 3-glycerophosphate pathway, which shows a high degree of specificity for saturated fatty acids at the *sn*-1 (3) position and for unsaturated fatty acids at the *sn*-2 position. In the liver, triacylglycerols can either be stored temporarily or incorporated into triacylglycerol-rich VLDL and released into the plasma. The triacylglycerol fatty acids of VLDL have the same fate as chylomicron triacylglycerol fatty acids. When VLDL triacylglycerols undergo lipolysis, the remaining triacylglycerol-depleted particle is called a VLDL remnant. These remnants are either removed directly by the liver or they are further metabolized in the vascular compartment to form low density lipoproteins (LDL).

Excretion. Fatty acids are generally catabolized entirely by oxidative processes from which the only excretion products are carbon dioxide and water. Small amounts of ketone bodies produced by fatty acid oxidation are excreted in urine. Fatty acids are present in the cells of the skin and intestine, thus small quantities are lost when these cells are sloughed.

Saturated Fatty Acids

Absorption. When saturated fatty acids are ingested along with fats containing appreciable amounts of unsaturated fatty acids, they are absorbed almost completely by the small intestine. In general, the longer the chain length of the fatty acid, the lower will be the efficiency of absorption. However, unsaturated fatty acids are well absorbed regardless of chain length. Studies with human infants have shown the absorption to be 75, 62, 92, and 94 percent of palmitic acid, stearic acid, oleic acid, and linoleic acid, respectively, from vegetable oils (Jensen et al., 1986). The absorption of palmitic acid and stearic acid from human milk is higher than from cow milk and vegetable oils (which are commonly used in infant formulas) because of the specific positioning of these long-chain saturated fatty acids at the *sn*-2 position of milk triacylglycerols (Carnielli et al., 1996a; Jensen, 1999). The intestinal absorption of palmitic acid and stearic acid from vegetable oils was 75 to 78 percent compared with 91 to 97 percent from fats with these fatty acids in the *sn*-2 position (Carnielli et al., 1996a). Still, absorption of stearic acid was over 90 percent complete in healthy adults when contained in triacylglycerols of mixed fatty acids (Bonanome and Grundy, 1989). Long-chain saturated fatty acids released into the lumen through the action of pancreatic lipase are less readily solubilized into mixed micelles than are unsaturated fatty acids; in the alkaline pH of the intestine they can form insoluble soaps with calcium and other divalent

cations and can be excreted (Carnielli et al., 1996a; Lucas et al., 1997; Tomarelli et al., 1968). Following absorption, long-chain saturated fatty acids are re-esterified along with other fatty acids into triacylglycerols and released in chylomicrons. Medium-chain saturated fatty acids (C8:0 and C10:0) are absorbed and transported bound to albumin as free fatty acids in the portal circulation and cleared by the liver. About two-thirds of lauric acid (C12:0) is transported with chylomicron triacylglycerols, whereas the remaining one-third enters the portal circulation as free fatty acids.

Metabolism. Pathways of oxidation of saturated fatty acids are similar to those for other types of fatty acids (see earlier section, "Total Fat"). Unoxidized stearic acid (9 to 14 percent) is rapidly desaturated and converted to the monounsaturated fatty acid, oleic acid (Emken, 1994; Rhee et al., 1997). For this reason, dietary stearic acid has metabolic effects that are closer to those of oleic acid rather than those of other long-chain saturated fatty acids. The saturated fatty acids, in contrast to *cis* mono- or polyunsaturated fatty acids, have a unique property in that they suppress the expression of LDL receptors (Spady et al., 1993). Through this action, dietary saturated fatty acids raise serum LDL cholesterol concentrations (Mustad et al., 1997).

Excretion. Saturated fatty acids, like other fatty acids, are generally completely oxidized to carbon dioxide and water.

cis-Monounsaturated Fatty Acids

Absorption. The absorption of *cis*-monounsaturated fatty acids (based on oleic acid data) is in excess of 90 percent in adults and infants (Jensen et al., 1986; Jones et al., 1985). The pathways of *cis*-monounsaturated fat digestion and absorption are similar to those of other fatty acids (see earlier section, "Total Fat").

Metabolism. Oleic acid, the major monounsaturated fatty acid in the body, is derived mainly from the diet. Small amounts also come from desaturation of stearic acid. Stable isotope tracer methods have shown that approximately 9 to 14 percent of dietary stearic acid is converted to oleic acid in vivo (Emken, 1994; Rhee et al., 1997). Based on the amount of stearic acid in the average diet (approximately 3 percent of energy), desaturation of dietary stearic acid is not a main source of oleic acid in the body. Oleic acid is oxidized, as are all other fatty acids, by β -oxidation. However, there is some evidence that oxidation of chylomicron-derived oleic acid is significantly greater than for palmitic acid (Schmidt et al.,

1999). The metabolic implications of the differential rates of oxidation of saturated, monounsaturated, and *cis* *n*-6 and *n*-3 fatty acids are not clear.

Excretion. Because oleic acid is highly absorbed, little is excreted. As for other fatty acids, the oxidation of monounsaturated fatty acids results in production of carbon dioxide and water.

n-6 Polyunsaturated Fatty Acids

Absorption. The digestion and absorption of *n*-6 fatty acids is efficient and occurs via the same pathways as that of other long-chain fatty acids (see earlier section, "Total Fat").

Metabolism. Both saturated and *n*-9 monounsaturated fatty acids can be synthesized from the carbon moieties of carbohydrate and protein. Mammalian cells do not have the enzymatic ability to insert a *cis* double bond at the *n*-6 position of a fatty acid chain, thus *n*-6 fatty acids are essential nutrients. The parent fatty acid of the *n*-6 series is linoleic acid. Studies using isotopically labeled linoleic acid have shown that adults and newborn infants can desaturate and elongate linoleic acid to form arachidonic acid (Emken et al., 1998, 1999; Salem et al., 1996; Sauerwald et al., 1997). The elongation of linoleic acid involves the sequential addition of two carbon units and desaturation involves insertion of a methylene-interrupted double bond towards the carboxyl terminus, thus preserving the position of the first *n*-6 double bond. These longer-chain, more polyunsaturated *n*-6 fatty acids are found primarily in membrane phospholipids, and since they can be formed only in animal cells, arachidonic acid is present in the diet only in animal tissue lipids.

Recent studies using stable isotopically labeled fatty acids to investigate the effect of gestational age and intrauterine growth on essential fatty acid desaturation and elongation have shown that the conversion of linoleic to arachidonic acid occurs as early as 26 weeks of gestation, and is in fact more active at earlier gestational ages (Uauy et al., 2000a). In addition to its role as a precursor to dihomo- γ -linolenic acid and arachidonic acid, linoleic acid has a specific role in acylceramides, which are important in maintaining the epidermal water barrier (Hansen and Jensen, 1985).

The 18 and 20 carbon *n*-9, *n*-6, and *n*-3 fatty acids compete for a common $\Delta 6$ and $\Delta 5$ desaturase. In vitro studies have shown the $\Delta 6$ desaturase enzymes preference occurs in the order $18:3n-3 > 18:2n-6 > 18:1n-9$ (Brenner, 1974; Castuma et al., 1977). The formation of arachidonic acid and *n*-3 fatty acid metabolites also appears to be inhibited by the products of the reaction and by high amounts of substrate. Thus, high intakes of *n*-3 fatty acids or

arachidonic and linoleic acids will reduce the efficiency of conversion of linoleic acid to arachidonic acid and α -linolenic acid to its products (Emken et al., 1994, 1998, 1999). For example, Emken and coworkers (1994) reported that an intake of 30 g/d of linoleic acid resulted in a 40 to 54 percent lower conversion of stable isotopically labeled linoleic and α -linolenic acid to their metabolites compared to an intake of 15 g/d in healthy men. High dietary intakes of n -3 fatty acids result in reduced tissue arachidonic acid concentrations and synthesis of arachidonic acid-derived eicosanoids, with consequent effects on the balance of n -6 and n -3 fatty acid-derived eicosanoids that are produced. The reduction in arachidonic acid-derived eicosanoids due to high n -3 fatty acid intake involves effects on pathways of eicosanoid formation, in addition to reducing concentrations of precursor arachidonic acid availability.

Both the rate of oxidation to carbon dioxide and water and the acylation into different lipids differ among fatty acids of different chain length and unsaturation. Arachidonic acid is primarily found in tissue phospholipids, rather than in triacylglycerols or cholesterol esters. Retroconversion of adrenic acid to arachidonic acid occurs through cleavage of a 2-carbon unit from the carboxyl end of the fatty acid and may be important in maintaining adequate tissue concentrations of arachidonic acid. Besides being elongated to longer-chain fatty acids, arachidonic acid is the precursor to a number of eicosanoids (prostaglandins, thromboxanes, and leukotienes) that are involved in platelet aggregation, hemodynamics, and coronary vascular tone, which can have an effect on the onset of atherogenesis and coronary infarction (Kinsella et al., 1990).

Excretion. n -6 Fatty acids are almost completely absorbed and are either incorporated into tissue lipids, utilized in eicosanoid synthesis, or oxidized to carbon dioxide and water. Small amounts are lost during sloughing of cells from skin and other epithelial membranes.

n -3 Polyunsaturated Fatty Acids

Absorption. The digestion and absorption of n -3 fatty acids is similar to that of other long-chain fatty acids.

Metabolism. Humans are unable to insert a double bond at the n -3 position (*cis* 15) of a fatty acid of 18 carbons in length, and thus require a dietary source of n -3 fatty acids. The n -3 fatty acids cannot be formed from saturated, n -9 monounsaturated, or n -6 polyunsaturated fatty acids. The parent fatty acid of the n -3 series is α -linolenic acid, which can be further metabolized by elongation and desaturation to longer-chain, more highly

unsaturated metabolites using the same pathway and enzymes as those used for the *n*-6 fatty acids. α -Linolenic acid is desaturated by $\Delta 6$ desaturase, elongated, and then desaturated by $\Delta 5$ desaturase to form EPA, which is the precursor for series 3 eicosanoids and series 5 leukotrienes. The pathway leading from EPA to more highly unsaturated fatty acids involves the addition of two 2-carbon units, then a second $\Delta 6$ desaturation, after which the 24-carbon-chain fatty acid is transported to the peroxisomes and converted to DHA through one step of β -oxidation (Sprecher et al., 1995; Voss et al., 1991). DHA is a component of membrane structural lipids that are enriched in certain phospholipids, such as the ethanolamine phosphoglycerides and phosphatidylserine in nervous tissue, retina, and spermatozoa. α -Linolenic acid is not known to have any specific functions other than to serve as a precursor for synthesis of EPA and DHA.

High dietary intakes of EPA and DHA result in decreased tissue concentrations of arachidonic acid and increased concentrations of EPA and DHA, respectively. This results in changes in the balance of eicosanoids synthesized from the *n*-6 and *n*-3 fatty acids. The ability to convert α -linolenic acid to EPA and DHA differs among mammalian species. Studies using isotopically labeled α -linolenic acid, however, have shown that adults and newborn infants can desaturate and elongate α -linolenic acid to form DHA (Carnielli et al., 1996b; Salem et al., 1996; Sauerwald et al., 1996, 1997; Uauy et al., 2000a; Vermunt et al., 2000). Recent studies with infants have shown that the rates of conversion of α -linolenic acid to DHA appear to be higher in preterm infants and decrease with increasing gestational age (Uauy et al., 2000a). These types of studies have also shown that high intakes of α -linolenic acid result in reduced conversion to DHA (Vermunt et al., 2000).

Whereas the retroconversion of arachidonic acid to maintain tissue arachidonic acid requires the removal of only a single 2-carbon unit, the retroconversion of DHA to EPA is more complex and involves the removal of the double bond at the $\Delta 4$ position, in addition to a 2-carbon unit. Supplementation with DHA is accompanied by an increase in EPA, which could be explained by retroconversion of DHA to EPA or by inhibition of further metabolism of EPA formed from α -linolenic acid (Brossard et al., 1996; Conquer and Holub, 1996; Nelson et al., 1997; Vidgren et al., 1997).

Excretion. *n*-3 Fatty acids are almost completely absorbed and either oxidized to carbon dioxide and water, incorporated into tissue lipids, or utilized in eicosanoid synthesis. Small amounts of *n*-3 fatty acids are lost during sloughing of skin and other epithelial cells.

Trans Fatty Acids

Absorption. As with other fatty acids, the coefficient of absorption of elaidic acid (18:1*t*) is about 95 percent (Emken, 1979). Studies in humans using pure triacylglycerols containing deuterated *cis* and *trans* octadecenoic acid isomers varying in melting point and double bond position suggest that the presence of *trans* double bonds in the fatty acyl chain has no measurable effect on efficiency of absorption (Emken, 1979, 1984).

Transport. *Trans* fatty acids are transported similarly to other dietary fatty acids and are distributed within the cholesteryl ester, triacylglycerol, and phospholipid fractions of lipoproteins (Vidgren et al., 1998). Platelet lipids also contain *trans* fatty acids and their composition reflects *trans* fatty acid intake, as do other tissues (except the brain) (Mensink and Hornstra, 1995).

Metabolism. The *trans* isomers of oleic acid and linoleic acid that are formed during partial hydrogenation of unsaturated vegetable oils have been suggested to have potential adverse effects on fetal and infant growth and development through inhibition of the desaturation of linoleic acid and α -linolenic acid to arachidonic acid and DHA, respectively (Koletzko, 1992; van Houwelingen and Hornstra, 1994). Many animal and in vitro studies, however, have involved much higher amounts of *trans* than all-*cis* polyunsaturated fatty acids (Hwang et al., 1982; Shimp et al., 1982). Other animal studies have suggested that the deleterious effects seen with high intakes of *trans* fatty acid do not occur with amounts comparable to those consumed in a normal human diet containing sufficient amounts of linoleic acid (Bruckner et al., 1982; Zevenbergen et al., 1988).

Available animal and human data indicate that adipose tissue *trans* fatty acid content reflects the content of the diet and that selective accumulation does not occur (Emken, 1984). More recent attention has been focused on validating the use of adipose *trans* fatty acid content as a measure of long-term dietary intake. In a study of Canadian individuals, Chen and colleagues (1995b) reported that adipose tissue *trans* fatty acid patterns, particularly those isomers found in partially hydrogenated vegetable fat, reflected dietary sources. Garland and coworkers (1998) also reported that adipose tissue *trans* fatty acid patterns correlated with intake and noted a stronger relationship with the isomers found in vegetable fat rather than animal fat. The authors cautioned that the later conclusion may have been due to the smaller between-person variability with animal versus vegetable *trans* fatty acid intake. In a letter to the editor regarding this study, Aro and Salminen (1998) suggested that the stronger correlation between adipose tissue *trans* fatty acid isomers found in hydrogenated vegetable fat

rather than animal fat may be attributable to different rates of metabolism of the *trans* isomers. Two groups have used adipose tissue *trans* fatty acid to corroborate dietary *trans* fatty acid intake derived from food frequency questionnaires and found a strong relationship (Lemaitre et al., 1998; London et al., 1991). Despite these observations, it should be noted that adipose tissue *trans* fatty acid profiles can be confounded by the retention of intermediate products of β -oxidation (Emken, 1995).

Excretion. *Trans* fatty acids are completely catabolized to carbon dioxide and water.

Clinical Effects of Inadequate Intakes

Total Fat

Impaired Growth. Dietary fat is a major source of body fuel. If intakes of fat, along with carbohydrate and protein, are inadequate to meet energy needs, the individual will be in negative energy balance. Depending on the severity and duration, this may lead to malnutrition or starvation. In an energy-sufficient diet, carbohydrate can replace fat as a source of energy. In some populations, fat intakes are very low and body weight and health are maintained by high intakes of carbohydrate (Bunker et al., 1996; Falase et al., 1973; Shintani et al., 2001). Clearly, humans have the ability to adapt metabolically to a wide spectrum of fat-to-carbohydrate intake ratios. In the short term, an isocaloric diet can be either very high or very low in fat with no obvious differences in health. The critical question therefore is, Are there optimal fat-to-carbohydrate ratios for long-term health, and if so, what are they? One potential concern over fat restriction is the potential for reduction in total energy intake, which is of particular relevance for infants and children, as well as during pregnancy when there is a relatively high energy requirement for both energy expenditure and for fetal development. Chapter 11 provides a detailed discussion on fat intake and growth.

Increased Risk of Chronic Diseases. Compared to higher fat intakes, low fat, high carbohydrate diets may modify the metabolic profile in ways that are considered to be unfavorable with respect to chronic diseases such as coronary heart disease (CHD) and diabetes (see Chapters 6 and 11). These changes include a reduction in high density lipoprotein cholesterol concentration, an increase in serum triacylglycerol concentration, and higher responses in postprandial glucose and insulin concentrations. This metabolic pattern has been associated with increased risk for CHD and type 2 diabetes

in intervention and prospective studies (see Chapter 11). Although changes in the metabolic profile do occur, strong evidence that low fat diets actually predispose to either CHD or diabetes does not exist. In fact, some populations that consume low fat diets and in which habitual energy intake is relatively high have a low prevalence of these chronic diseases (Falase et al., 1973; Shintani et al., 2001). Similarly, populations with high fat diets (i.e., ≥ 40 percent of energy) and a low prevalence of chronic diseases often include people who engage in heavy physical labor, are lean, and have a low family history of chronic diseases. Conversely, in sedentary populations, such as that of the United States where overweight and obesity are common, high carbohydrate, low fat diets induce changes in lipoprotein and glucose/insulin metabolism in ways that could raise risk for chronic diseases (see Chapter 11). Available prospective studies have not concluded whether low fat, high carbohydrate diets provide a health risk in the North American population.

Chronic nonspecific diarrhea in children has been suggested as a potential adverse effect of low fat diets. It is considered a disorder of intestinal motility that may improve with an increase in dietary fat intake in order to slow gastric emptying and alter intestinal motility (Cohen et al., 1979). Detailed discussion on fat intake and risk of chronic disease is provided in Chapter 11.

n-6 Polyunsaturated Fatty Acids

Certain polyunsaturated fatty acids were first identified as being essential in rats fed diets almost completely devoid of fat (Burr and Burr, 1929). Subsequently, studies in infants and children fed skimmed cow milk (Hansen et al., 1958, 1963) and patients receiving parenteral nutrition without an adequate source of essential fatty acids (Collins et al., 1971; Holman et al., 1982; Paulsrud et al., 1972) demonstrated clinical symptoms of a deficiency in humans. Because adipose tissue lipids in free-living, healthy adults contain about 10 percent of total fatty acids as linoleic acid, biochemical and clinical signs of essential fatty acid deficiency do not appear during dietary fat restriction or malabsorption when they are accompanied by an energy deficit. In this situation, release of linoleic acid and small amounts of arachidonic acid from adipose tissue reserves may prevent development of essential fatty acid deficiency. However, during parenteral nutrition with dextrose solutions, insulin concentrations are high and mobilization of adipose tissue is prevented, resulting in development of the characteristic signs of essential fatty acid deficiency. Studies on patients given fat-free parenteral feeding have provided great insight into defining levels at which essential fatty acid deficiency may occur. Without intervention, these patients develop clinical signs of a deficiency

in 2 to 4 weeks (Fleming et al., 1976; Goodgame et al., 1978; Jeppesen et al., 1998; Riella et al., 1975). In rapidly growing infants, feeding with milk containing very low amounts of *n*-6 fatty acids results in characteristic signs of an essential fatty acid deficiency and elevated plasma triene:tetraene ratios (see "*n*-6:*n*-3 Polyunsaturated Fatty Acid Ratio").

When dietary essential fatty acid intake is inadequate or absorption is impaired, tissue concentrations of arachidonic acid decrease, inhibition of the desaturation of oleic acid is reduced, and synthesis of eicosatrienoic acid from oleic acid increases. The characteristic signs of deficiency attributed to the *n*-6 fatty acids are scaly skin rash, increased transepidermal water loss, reduced growth, and elevation of the plasma ratio of eicosatrienoic acid:arachidonic acid (20:3*n*-9:20:4*n*-6) to values greater than 0.4 (Goodgame et al., 1978; Holman, 1960; Jeppesen et al., 2000; Mascioli et al., 1996; O'Neill et al., 1977). Other studies have utilized a ratio of 0.2 as indicative of an essential fatty acid deficiency (Holman et al., 1991; Jeppesen et al., 1998). In addition to the clinical signs mentioned above, essential fatty acid deficiency in special populations has been linked to hematologic disturbances and diminished immune response (Bistrian et al., 1981; Boissonneault and Johnston, 1983). Further discussion on this topic is included in "Findings by Life Stage and Gender Group—*n*-6 Polyunsaturated Fatty Acids."

n-3 Polyunsaturated Fatty Acids

Tissue levels of arachidonic acid, as well as the amounts of arachidonic acid and EPA-derived eicosanoids that are formed, have important effects on many physiological processes (e.g., platelet aggregation, vessel wall constriction, and immune cell function) via the biosynthesis of eicosanoids. Thus, the amount of *n*-3 fatty acids and their effects on arachidonic acid metabolism are relevant to many chronic diseases. EPA also appears to have specific effects on fatty acid metabolism, resulting in inhibition of hepatic triacylglycerol synthesis and VLDL secretion (Berge et al., 1999; Wong and Nestel, 1987). DHA, on the other hand, is highly enriched in specific phospholipids of the retina and nonmyelin membranes of the nervous system.

Studies in rodents and nonhuman primates have consistently demonstrated that prolonged feeding with diets containing very low amounts of α -linolenic acid result in reductions of visual acuity thresholds and electroretinogram A and B wave recordings, which were prevented when α -linolenic acid was included in the diet (Anderson et al., 1974; Benolken et al., 1973; Bourre et al., 1989; Neuringer et al., 1984, 1986; Wheeler et al., 1975). A variety of changes in learning behaviors in animals fed α -linolenic acid-deficient diets have also been reported (Innis, 1991). These studies have

involved feeding oils such as safflower oil, which contains less than 0.1 percent α -linolenic acid and is high in linoleic acid, as the sole source of fat for prolonged periods. The reduction in visual function is accompanied by decreased brain and retina DHA with an increase in docosapentaenoic acid (DPA, 22:5 n -6). The compensatory increase in 22 carbon chain n -6 fatty acids results in maintenance of the total amount of n -6 and n -3 polyunsaturated fatty acids in neural tissue. DPA is formed from linoleic acid by similar desaturation and elongation steps used in the synthesis of DHA from α -linolenic acid. However, α -linolenic acid is clearly handled differently from linoleic acid. For example, rates of β -oxidation of α -linolenic acid are much higher than for linoleic acid (Clouet et al., 1989). This may suggest that immaturity or reduced enzyme activity is unlikely to explain lower DHA in the brain of young animals fed diets with low amounts of α -linolenic acid, and that DHA has specific metabolic functions that cannot be accomplished by DPA despite its structural similarity. Stable isotope studies have shown that infants can convert linoleic acid to arachidonic acid and α -linolenic acid to DHA (Carnielli et al., 1996b; Salem et al., 1996; Sauerwald et al., 1996, 1997; Uauy et al., 2000a), with the rate of conversion apparently higher in infants of younger gestational ages (Uauy et al., 2000a).

Unlike essential fatty acid deficiency (n -6 and n -3 fatty acids), plasma eicosatrienoic acid (20:3 n -9) remains within normal ranges and skin atrophy and scaly dermatitis are absent when the diet is deficient in only n -3 fatty acids. Tissue concentrations of 22-carbon chain n -6 fatty acids increase, and DHA concentration decreases with a prolonged dietary deficiency of n -3 fatty acids accompanied by adequate n -6 fatty acids. Currently, there are no accepted plasma n -3 fatty acid or n -3 fatty acid-derived eicosanoid concentrations for indicating impaired neural function or impaired health endpoints. Further discussion on this topic is included in the next section.

EVIDENCE CONSIDERED FOR ESTIMATING THE REQUIREMENTS FOR TOTAL FAT AND FATTY ACIDS

Total Fat

Clinical endpoints of fat intake are trends, rather than defined endpoints, and therefore cannot be used to set an Estimated Average Requirement (EAR). The endpoints that strongly predict the relation of total fat intake to the development of chronic disease have been identified and are discussed in Chapter 11 for estimating Acceptable Macronutrient Distribution Ranges (AMDRs).

Growth

Because the amount of fat in the diet can have an impact on energy intake, a number of studies have been conducted to determine if diets containing less than 30 percent of energy from fat can impair growth of children (Boulton and Magarey, 1995; Foman et al., 1976; Lagström et al., 1999; Lapinleimu et al., 1995; Niinikoski et al., 1997a, 1997b; Obarzanek et al., 1997; Shea et al., 1993; Uauy et al., 2000b; Vobecky et al., 1995). These studies showed no effect of the level of dietary fat on growth when energy intake is adequate. Chapter 11 provides further discussion on this topic.

Fat Balance (Maintenance of Body Weight)

Because fat is an important source of energy, studies have been conducted to ascertain whether dietary fat influences energy expenditure and the amount of fat needed in the diet to achieve fat balance and therefore maintain body weight. These studies demonstrated that the amount of fat in the diet does not affect energy expenditure and thus the amount of energy required to maintain body weight (Hill et al., 1991; Leibel et al., 1992). Chapter 11 provides further discussion on this topic.

Saturated Fatty Acids

Saturated fatty acids are a potential fuel source for the body. In addition, they are important structural fatty acids for cell membranes and other functions and therefore are essential for body functions. These fatty acids, however, can be synthesized as needed for these functions from other fuel sources and have not been associated with any beneficial role in preventing chronic disease. Consequently, saturated fatty acids are not essential in the diet.

cis-Monounsaturated Fatty Acids

Monounsaturated fatty acids are a potential fuel source for the body and are a critical structural fatty acid for cell membranes and other functions. Monounsaturated fatty acids undoubtedly are required for many body functions. Nevertheless, monounsaturated fatty acids can be biosynthesized from other fuel sources and therefore are not essential in the diet.

n-6 Polyunsaturated Fatty Acids

Clinical signs of essential fatty acid deficiency are generally only found in patients with chronic fat malabsorption on parenteral nutrition and without an enteral or parenteral source of polyunsaturated fat. Early signs of essential fatty acid deficiency include rough and scaly skin, which if left untreated, develops into dermatitis (Jeppesen et al., 1998). In studies of patients with dermatitis who were receiving parenteral nutrition, the ratio of eicosatrienoic acid:arachidonic acid ($20:3n-9:20:4n-6$) in plasma was elevated. As described earlier, when present in adequate amounts, linoleic acid is converted to arachidonic acid through a multi-step process involving $\Delta 6$ and $\Delta 5$ desaturases (see Figure 8-1); however, in the absence of linoleic acid, $\Delta 6$ and $\Delta 5$ desaturases convert oleic acid to eicosatrienoic acid. The increase in eicosatrienoic acid concentration, which occurs in the absence of $n-6$ fatty acids or the combined absence of $n-6$ and $n-3$ fatty acids, led Holman (1960) to define a plasma triene:tetraene ratio of greater than 0.4 as evidence of essential fatty acid deficiency. More recently, a lower threshold of greater than 0.2 has been suggested (Holman et al., 1979; Jeppesen et al., 1998; Mascioli et al., 1996) because the average ratio was found to be 0.1 ± 0.08 (standard deviation) in populations of normal $n-6$ fatty acid status. Optimal plasma or tissue lipid concentrations of linoleic acid, arachidonic acid, and other $n-6$ fatty acids or the ratios of certain $n-6:n-3$ fatty acids have not been established.

Because the $n-6$ fatty acid intake is generally well above the levels needed to maintain a triene:tetraene ratio below 0.2 (even for very low fat diets), data on $n-6$ fatty acid requirements from traditional metabolic feeding studies are not available. Instead, studies with patients on total parenteral nutrition (TPN) solutions that contained very low amounts or were completely devoid of $n-6$ fatty acids have been used. In these studies, after developing an essential fatty acid deficiency, patients were treated with linoleic acid. Several case reports, small studies of two or three patients in which varying feeding designs were employed, or larger studies of patients with $n-6$ fatty acid deficiency caused by TPN have been documented (Barr et al., 1981; Collins et al., 1971; Goodgame et al., 1978; Jeppesen et al., 1998; Mascioli et al., 1979; Meng, 1983; Richardson and Sgoutas, 1975; Riella et al., 1975; Siguel et al., 1986; Wene et al., 1975; Wong and Deitel, 1981). These studies observed symptoms such as rash, scaly skin, and ectopic dermatitis; reduced serum tetraene concentrations, increased serum triene concentration; and a triene:tetraene ratio greater than 0.4 after 2 to 4 weeks of TPN. Because of the lack of data on the $n-6$ fatty acid requirement in healthy individuals, an EAR cannot be set based on correction of a deficiency.

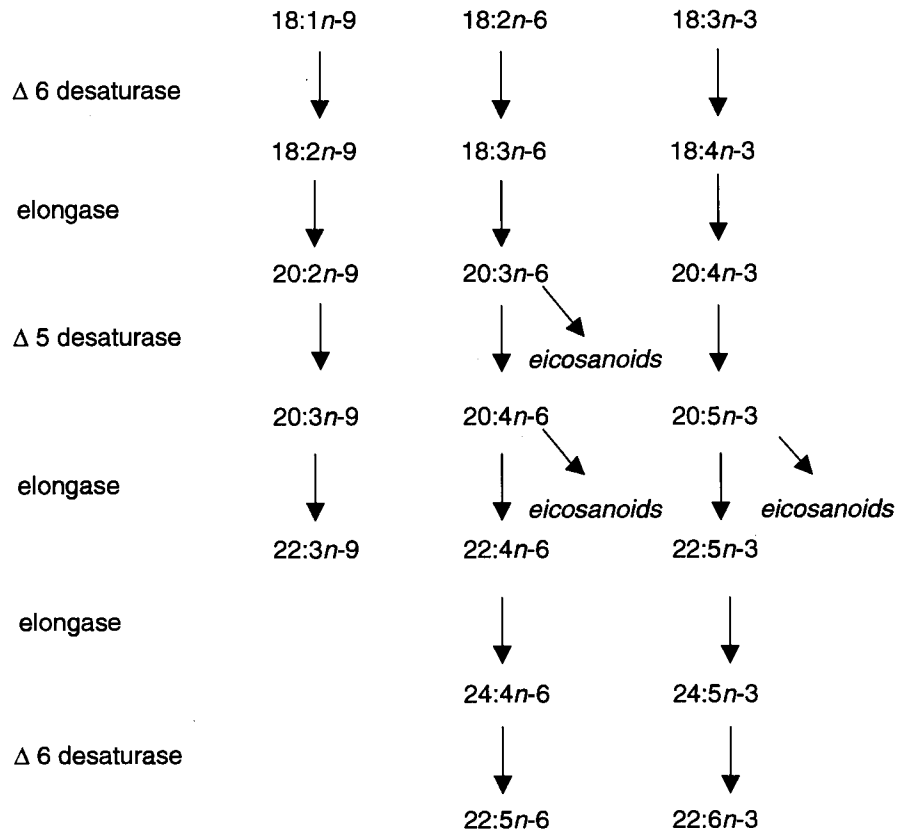


FIGURE 8-1 Biosynthesis of long-chain fatty acids.

n-3 Polyunsaturated Fatty Acids

n-3 Polyunsaturated Fatty Acid Deficiency

Some evidence for the essentiality of *n-3* fatty acids in humans can be drawn from case reports of patients receiving parenteral nutrition with intravenous lipids containing an emulsion of safflower oil, which is very low in α -linolenic acid and high in linoleic acid. Biochemical changes of *n-3* fatty acid deficiency include a decrease in plasma and tissue docosahexaenoic acid (DHA) concentrations. There is no accepted cut-off concentration of plasma or tissue DHA concentrations below which functions ascribed to *n-3* fatty acids, such as visual or neural function, are impaired. Similarly, there are no accepted normal ranges for eicosapentaenoic acid (EPA) with respect to synthesis of EPA-derived eicosanoids or regulation of arachidonic acid metabolism and its eicosanoid metabolites, nor are there accepted clinical functional endpoints such as immune response.

Dietary or intravenous supplementation with oils containing α -linolenic acid, such as soybean oil, has been shown to increase red blood cell and plasma phospholipid DHA concentration in hospitalized patients with a long history of dietary n -3 fatty acid restriction (Bjerve et al., 1987a, 1987b; Holman et al., 1982). Sensory neuropathy and visual problems in a young girl given parenteral nutrition with an intravenous lipid emulsion containing only a small amount of α -linolenic acid were corrected when the emulsion was changed to one containing generous amounts of α -linolenic acid (Holman et al., 1982). Nine patients with an n -3 fatty acid deficiency had scaly and hemorrhagic dermatitis, hemorrhagic folliculitis of the scalp, impaired wound healing, and growth retardation (Bjerve, 1989). The possibility of other nutrient deficiencies, such as vitamin E and selenium, has been raised (Anderson and Connor, 1989; Meng, 1983). A series of papers have described low tissue n -3 fatty acid concentrations in nursing home patients fed by gastric tube for several years with a powdered diet formulation that provided about 0.5 to 0.6 percent of energy (0.65 to 0.86 g) as linoleic acid, and 0.02 percent of energy (30 to 50 mg) as α -linolenic acid (Bjerve et al., 1987a, 1987b). Skin lesions were resolved following supplementation with cod liver oil and soybean oil or ethyl linolenate (Bjerve et al., 1987a, 1987b). Concurrent deficiency of both n -6 and n -3 fatty acids in these patients, as in studies of patients supported by lipid-free parenteral nutrition, limits interpretation of the specific problems caused by inadequate intakes of n -3 fatty acids. Supplementation with cod liver oil and soybean oil, or feeding with a formula providing linoleic acid and α -linolenic acid or ethyl α -linolenic acid for 14 days, increased red blood cell arachidonic acid and DHA concentrations and gave some resolution of skin signs (Bjerve et al., 1987a, 1987b). Because of the lack of data on the n -3 fatty acid requirement in healthy individuals, an EAR cannot be set based on correction of a deficiency.

Growth and Neural Development

The membrane lipids of brain gray matter and the retina contain very high concentrations of DHA, particularly in the amino phospholipids phosphatidylethanolamine and phosphatidylserine. In these tissues, the concentration of DHA can exceed 50 percent of the fatty acids resulting in the presence of di-DHA phospholipid species. During n -3 fatty acid deficiency, DHA is tenaciously retained, thus most animal studies investigating the importance of n -3 fatty acids have used rats deprived of n -3 fatty acids for two or more generations. Small amounts of DHA are also present in cell membranes throughout the body. In these tissues, the phospholipid sn -1 chain is usually a saturated fatty acid (e.g., 16:0) and DHA is found on the sn -2 position. The developing brain accumulates large amounts of DHA

during pre- and postnatal development and this accumulation continues throughout the first two years after birth (Martinez, 1992). Evidence from autopsy analysis indicates that accumulation of DHA in the retina is complete by term birth (Martinez et al., 1988). Due to the accumulation of DHA during brain growth, the developing brain is more susceptible to *n*-3 fatty acid deficiency than the mature brain. However, the presence of DHA within the membrane hydrophobic interior can influence membrane order (fluidity), thickness, domain size, hydration, and permeability and activity of associated proteins and ion channels. Unesterified DHA also regulates the expression of a variety of genes and influences cell signaling mechanisms (Salem et al., 2001; Sinclair et al., 2000). Animal studies have shown that feeding a diet very low in α -linolenic acid results in reduced brain and retina DHA concentration, which is accompanied by reduced visual function and behavior in learning tasks (Benolken et al., 1973; Bourre et al., 1989; Neuringer et al., 1984; Wheeler et al., 1975). The decrease in DHA concentration in the brain and retina is compensated for by an increase in the *n*-6 fatty acid docosapentaenoic acid, and this leads to maintenance of the total polyunsaturated fatty acid content of the membrane. Reduced growth or changes in food intake have not been noted in the extensive number of studies in animals, including nonhuman primates fed for extended periods on otherwise adequate diets lacking *n*-3 fatty acids.

The essential role of α -linolenic acid appears to be its role as precursor for synthesis of EPA and DHA. Thus, the dietary *n*-3 fatty acid requirement involves the activity of the desaturase enzymes and factors that influence the desaturation of α -linolenic acid in addition to the amount of the *n*-3 fatty acid. The questions of whether term gestation infants can form DHA, or if DHA is required in the infant diet, has been studied extensively. Activity of $\Delta 6$ and $\Delta 5$ desaturases has been demonstrated in human fetal tissue from as early as 17 to 18 weeks of gestation (Chambaz et al., 1985; Rodriguez et al., 1998), and stable isotope studies have confirmed that preterm and term infants are able to convert α -linolenic acid to DHA (Carnielli et al., 1996b; Salem et al., 1996; Sauerwald et al., 1996, 1997; Uauy et al., 2000a). Furthermore, the ability to convert α -linolenic acid appears to be greater in premature infants than in older term infants (Uauy et al., 2000a), although variability among infants is large. Current information from stable isotope tracer studies does not provide quantitative whole body or organ data on the conversion of α -linolenic acid to DHA, whether the rate of conversion can meet the needs of the developing brain for DHA, or the effect of varying linoleic and α -linolenic acid intakes and ratios on conversion. Experimental studies suggest that the eye and certain brain cells, such as astrocytes, are able to synthesize DHA from α -linolenic acid (Moore et al., 1991; Wetzel et al., 1991). The contribution of synthesis of DHA in the brain and retina to the accumulation of

DHA in these organs is not known. In vivo studies, however, have shown that the brain does take up DHA from plasma (de la Presa Owens and Innis, 1999; Greiner et al., 1997).

A large number of clinical trials have been completed comparing growth, as well as measures of visual, motor, and mental development, in term infants fed formula with no DHA or with addition of DHA to approximate the amount in human milk. Some have included arachidonic acid or γ -linolenic acid (18:3 n -6), the Δ 6 desaturase product of linoleic acid. The results of these trials are summarized in Table 8-1. Several aspects of design are important in evaluating these studies. These include a prospective, double-blind design with a sufficient number of infants randomized to control for the multiple genetic, environmental, and dietary factors that influence infant development and to detect meaningful treatment effects (Gore, 1999; Morley, 1998); the amount and balance of linoleic and α -linolenic acid; the duration of supplementation; the age at testing and tests used; and the physiological significance of any statistical differences found. None of the studies in Table 8-1 reported differences in growth among infants fed formulas with DHA added.

Recent large, randomized trials did not find differences in visual evoked potential, visual acuity, or tests of mental and psychomotor development through at least the first 18 months in term infants fed formulas supplemented with DHA or DHA plus arachidonic acid (Auestad et al., 1997, 2001; Lucas et al., 1999; Scott et al., 1998). These studies used formulas with at least 1.1 percent α -linolenic acid and had linoleic: α -linolenic acid ratios close to 10:1. In the study by Scott and coworkers (1998), indices of early vocabulary development were lower in infants fed formula with DHA, but not in those fed formulas lacking DHA and arachidonic acid or containing both DHA and arachidonic acid. Birch and coworkers (1998, 2000) reported better visual evoked potential, but not visual acuity, and higher Bayley mental developmental indices scores in infants fed formulas with DHA or DHA plus arachidonic acid than in infants fed standard formula. Carlson and coworkers (1996a) on the other hand, found higher visual acuity at 2 months, but not at 4, 6, 9, or 12 months, in infants fed formula with DHA and arachidonic acid. Early studies by Makrides and colleagues (1995) reported better visual evoked potential acuity in infants fed formula with 0.36 percent DHA than infants given no dietary DHA. However, this group did not confirm this finding in subsequent studies with formulas containing 0.34 or 0.35 percent DHA (Makrides et al., 2000b). In addition, greater problem-solving ability has been reported among infants fed formula with DHA and arachidonic acid than in infants fed standard formula (Willatts et al., 1998).

The effect of low n -6: n -3 ratios (high n -3 fatty acids) on arachidonic acid metabolism is also of concern in growing infants. Several studies in

premature infants have reported an association between feeding *n*-3 long-chain fatty acids in the absence of arachidonic acid and reduced growth (Carlson et al., 1992, 1993, 1996b; Ryan et al., 1999). Scott and coworkers (1998) reported lower indices of language development in term infants fed formula with DHA, although not in infants fed formula with both DHA and arachidonic acid or with no DHA and arachidonic acid. Human milk from women in the United States and Canada following usual diets contains both arachidonic acid and DHA, usually in the range of 1:1 to 2:1. No evidence of reduced growth or outcome on developmental tests have been reported for infants fed formulas with both arachidonic acid and DHA in amounts similar to that contained in human milk. Infants fed formula with a ratio of linoleic: α -linolenic acid of 4.8:1 and no arachidonic acid had lower growth, as well as lower plasma arachidonic acid status, than infants fed a formula with a ratio of 44:1 (Jensen et al., 1997), and no differences in growth were found between infants fed formulas containing linoleic: α -linolenic acid ratios of 9.7:1 and 18.2:1. Additionally, no differences in growth were found among infants fed formulas with 1.7 or 3.3 percent α -linolenic acid with linoleic: α -linolenic acid ratios of 10:1 or 5:1, respectively (Makrides et al., 2000a).

In conclusion, randomized clinical studies on growth or neural development with term infants fed formulas currently yield conflicting results on the requirements for *n*-3 fatty acids in young infants, but do raise concern over supplementation with long-chain *n*-3 fatty acids without arachidonic acid. For these reasons, growth and neural development could not be used to set an EAR.

Trans Fatty Acids and Conjugated Linoleic Acid

Small amounts of *trans* fatty acids and conjugated linoleic acid are present in all diets. They can serve as a source of fuel energy for the body. However, there are no known requirements for *trans* fatty acids and conjugated linoleic acid for specific body functions.

FACTORS AFFECTING THE REQUIREMENTS

Fat Absorption and Aging

Aging in humans has been associated with a decrease in liver size and hepatic blood flow, slightly decreased serum albumin concentrations, and normal routine liver chemistries (Russell, 1992). Pancreatic secretion after initial stimulation with either secretin or pancreozymin is not diminished with age (Bartoš and Groh, 1969). Similarly, 72-hour fecal fat excretion in response to a dietary fat challenge in young (19 to 44 years of age) and old

TABLE 8-1 Randomized Studies of *n*-3 Fatty Acids and Neural and Visual Development in Full-Term, Formula-Fed Infants

Reference	Study Population ^a	Test/Age ^b	Fatty Acid ^c
Agostoni et al., 1995	<i>n</i> = 29 formula <i>n</i> = 29 formula + LC-PUFA	Brunet-Lézine psychomotor development test 4 mo	18:2 <i>n</i> -6 18:3 <i>n</i> -3 18:3 <i>n</i> -6 (GLA) 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)
Makrides et al., 1995	<i>n</i> = 14 formula <i>n</i> = 12 formula + LC-PUFA	VEP acuity 16, 30 wk	18:2 <i>n</i> -6 18:3 <i>n</i> -3 18:3 <i>n</i> -6 (GLA) 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)
Carlson et al., 1996a	<i>n</i> = 20 formula <i>n</i> = 19 formula + DHA + AA	Visual acuity 2, 4, 6, 9, 12 mo	18:2 <i>n</i> -6 18:3 <i>n</i> -3 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)
Agostoni et al., 1997	<i>n</i> = 30 formula <i>n</i> = 26 formula + LC-PUFA	DQ 24 mo	18:2 <i>n</i> -6 18:3 <i>n</i> -3 18:3 <i>n</i> -6 (GLA) 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)
Auestad et al., 1997	<i>n</i> = 45 formula <i>n</i> = 43 formula + DHA <i>n</i> = 46 formula + DHA + AA	Sweep VEP 2, 4, 6, 9, 12 mo Visual acuity 2, 4, 6, 9, 12 mo	 18:2 <i>n</i> -6 18:3 <i>n</i> -3 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)
Jensen et al., 1997	<i>n</i> = 20 each group	VER 4 mo	18:2 <i>n</i> -6 18:3 <i>n</i> -3 18:2 <i>n</i> -6:18:3 <i>n</i> -3 ratio
Birch et al., 1998	<i>n</i> = 21 formula <i>n</i> = 20 formula + DHA <i>n</i> = 19 formula + DHA + AA	Sweep VEP acuity 6, 17, 26, 52 wk Visual acuity 6, 17, 26, 52 wk	 18:2 <i>n</i> -6 18:3 <i>n</i> -3 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)

Fatty Acid Content (% of fatty acids)		Results		
<u>Formula</u>	<u>Formula + LC-PUFA</u>	Infants consuming formula supplemented with LC-PUFA scored significantly higher than standard formula group		
11.1	10.8			
0.70	0.73			
—	0.30			
—	0.44			
—	0.30			
<u>Formula</u>	<u>Formula + LC-PUFA</u>	VEP acuity better in infants fed supplemented formula than in infants fed standard formula		
16.79	17.44			
1.58	1.52			
0.05	0.27			
—	0.01			
	0.36			
<u>Formula</u>	<u>Formula + DHA + AA</u>	Infants fed formula supplemented with DHA + AA had higher visual acuity than infants fed standard formula at 2 mo, but not at 4, 6, 9, or 12 mo		
21.9	21.8			
2.2	2.0			
—	0.43			
—	0.10			
<u>Formula</u>	<u>Formula + LC-PUFA</u>	No differences in DQ values		
11.1	10.8			
0.70	0.73			
—	0.30			
—	0.44			
—	0.30			
<u>Formula</u>	<u>Formula + DHA</u>	<u>Formula + DHA + AA</u>	No differences in VEP or visual acuity	
21.9	20.7	21.7		
2.2	1.9	1.9		
—	—	0.43		
—	0.23	0.12		
<u>Formula #1</u>	<u>#2</u>	<u>#3</u>	<u>#4</u>	No differences in VER Infants fed formula with a ratio of 4.8 weighed less than infants fed formula with a ratio of 44
17.6	17.3	16.5	15.6	
0.4	0.95	1.7	3.2	
44.0	18.2	9.7	4.8	
<u>Formula</u>	<u>Formula + DHA</u>	<u>Formula + DHA + AA</u>	Sweep VEP acuity better in infants fed supplemented formulas than in infants fed standard formula at 6, 17, and 52 wk, but not 26 wk Visual acuity not different between groups	
14.6	15.1	14.9		
1.49	1.54	1.53		
—	0.02	0.72		
—	0.35	0.36		

continued

TABLE 8-1 Continued

Reference	Study Population ^a	Test/Age ^b	Fatty Acid ^c
Jørgensen et al., 1998	<i>n</i> = 11 formula <i>n</i> = 12 formula + DHA <i>n</i> = 14 formula + DHA + GLA	Sweep VEP acuity 4 mo	18:2 <i>n</i> -6 18:3 <i>n</i> -3 18:3 <i>n</i> -6 (GLA) 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)
Scott et al., 1998	<i>n</i> = 42-45 formula <i>n</i> = 33-43 formula + DHA <i>n</i> = 38-46 formula + DHA + AA	Bayley scales of infant development 12 mo MacArthur communicative development 14 mo	18:2 <i>n</i> -6 18:3 <i>n</i> -3 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)
Lucas et al., 1999	<i>n</i> = 125 formula <i>n</i> = 125 formula + LC-PUFA	Bayley scales of infant development 18 mo	18:2 <i>n</i> -6 18:3 <i>n</i> -3 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)
Makrides et al., 2000a	<i>n</i> = 30 10:1 formula <i>n</i> = 28 5:1 formula	VEP acuity 16, 34 wk	18:2 <i>n</i> -6 18:3 <i>n</i> -3
Makrides et al., 2000b	<i>n</i> = 21 formula <i>n</i> = 23 formula + DHA <i>n</i> = 24 formula + DHA + AA	VEP acuity 16, 34 wk Bayley scales of infant development 12, 24 mo	18:2 <i>n</i> -6 18:3 <i>n</i> -3 20:4 <i>n</i> -6 (AA) 22:6 <i>n</i> -3 (DHA)

^a LC-PUFA = long chain polyunsaturated fatty acids.

^b VEP = visual evoked potential, DQ = developmental quotient, VER = visual evoked response.

(70 to 91 years of age) individuals suggests little change in the capacity to absorb fat (Arora et al., 1989). The ratio of mean surface area to volume of jejunal mucosa has been reported not to differ between young and old individuals (Corazza et al., 1986). Total gastrointestinal transit time appears to be similar between young and elderly individuals (Brauer et al.,

Fatty Acid Content (% of fatty acids)			Results
<u>Formula</u>	Formula + <u>DHA</u>	Formula + <u>DHA + GLA</u>	No differences in VEP acuity
12.01	11.95	12.67	
1.20	1.20	1.17	
—	—	0.54	
—	0.06	0.06	
—	0.32	0.32	
<u>Formula</u>	Formula + <u>DHA</u>	Formula + <u>DHA + AA</u>	No differences in mental and psychomotor development
21.9	20.7	21.7	Vocabulary production and comprehension lower in the formula + DHA group
2.2	1.9	1.9	
—	—	0.43	
—	0.23	0.12	
<u>Formula</u>	<u>Formula + LC-PUFA</u>		No differences in mental and psychomotor development
12.4	15.9		
1.1	1.4		
—	0.30		
—	0.32		
<u>10:1 Formula</u>	<u>5:1 Formula</u>		No differences in VEP acuity
16.9	16.6		
1.7	3.3		
<u>Formula</u>	Formula + <u>DHA</u>	Formula + <u>DHA + AA</u>	No differences in VEP acuity or Bayley scales of mental and psychomotor development
16.8	16.8	16.6	
1.5	1.2	1.0	
—	—	0.34	
—	0.35	0.34	

^c GLA = γ -linolenic acid, AA = arachidonic acid, DHA = docosahexaenoic acid.

1981). Documented changes with age may be confounded by the inclusion of a subgroup with clinical disorders (e.g., atrophic gastritis). The presence of bile salt-splitting bacteria normally present in the small intestine of humans is of potential significance to fat absorption. No evidence of bacterial overgrowth has been reported in older individuals (Arora et

al., 1989). In addition, increases in fat malabsorption have not been demonstrated in normal elderly compared to younger individuals (Russell, 1992).

Exercise

Imposed physical activity decreased the magnitude of weight gain in nonobese volunteers given access to high fat diets (60 percent of energy) (Murgatroyd et al., 1999). In the exercise group, energy and fat balances (fat intake + fat synthesis – fat utilization) were not different from zero. Thus, high fat diets may cause positive fat balance, and therefore weight gain, only under sedentary conditions. These results are consistent with epidemiological evidence that show interactions between dietary fat, physical activity, and weight gain (Sherwood et al., 2000). Higher total fat diets can probably be consumed safely by active individuals while maintaining body weight. Although in longitudinal studies of weight gain, where dietary fat predicts weight gain independent of physical activity, it is important to note that physical activity may account for a greater percentage of the variance in weight gain than does dietary fat (Hill et al., 1989). Another endpoint that merits consideration is physical performance. High fat diets (69 percent of energy) do not appear to compromise endurance in trained athletes (Goedecke et al., 1999); however, athletes may not be able to train as effectively on short-term (less than 6 days) intakes of a high fat diet as on a high carbohydrate diet (Helge, 2000). This effect on training was not observed following long-term adaptation of high fat diets.

Genetic Factors

Studies of the general population may underestimate the importance of dietary fat in the development of obesity in subsets of individuals. Some data indicate that genetic predisposition may modify the relationship between diet and obesity (Heitmann et al., 1995). Additionally, some individuals with relatively high metabolic rates appear to be able to consume high fat diets (44 percent of energy) without obesity (Cooling and Blundell, 1998). Intervention studies have shown that those individuals susceptible to weight gain and obesity appear to have an impaired ability to increase fat oxidation when challenged with high fat meals and diets (Astrup et al., 1994; Raben et al., 1994). Animal studies show that there are important gene and dietary fat interactions that influence the tendency to gain excessive weight on a high fat diet (West and York, 1998). Once these genes are identified, further studies in humans will be feasible.

Alcohol

Alcohol is metabolized to acetylcoenzyme A in the liver and can enter all normal pathways for acetate metabolism, including the synthesis of fatty acids. The formation of nicotinamide adenine dinucleotide, resulting from ethanol oxidation, serves as a cofactor for fatty acid biosynthesis (Eisenstein, 1982). Similar to carbohydrate, alcohol consumption creates a shift in postprandial substrate utilization to reduce the oxidation of fatty acids (Schutz, 2000). Significant intake of alcohol (23 percent of energy) can depress fatty acid oxidation to a level equivalent to storing as much as 74 percent as fat (Murgatroyd et al., 1996). If the energy derived from alcohol is not utilized, the excess is stored as fat (Suter et al., 1992).

Interaction of n-6 and n-3 Fatty Acid Metabolism

The *n*-6 and *n*-3 unsaturated fatty acids are believed to be desaturated and elongated using the same series of desaturase and elongase enzymes (see Figure 8-1). The rate-limiting steps are the desaturases, rather than the elongase, enzymes. In vitro, the $\Delta 6$ desaturase shows clear substrate preference in the following order: α -linolenic acid > linoleic acid > oleic acid (Brenner, 1974). In addition, the formation of docosahexaenoic acid (DHA) from tetracosapentenoic acid (24:5*n*-3) involves a $\Delta 6$ desaturation to 24:6*n*-3 and then β -oxidation to yield 22:6*n*-3 (DHA) (Sprecher, 1992). It is not known if these are the $\Delta 6$ desaturases that are responsible for metabolism of linoleic acid and α -linolenic acid or a different enzyme (Cho et al., 1999). Many studies, primarily in laboratory animals, have provided evidence that the balance of linoleic and α -linolenic acid is important in determining the amounts of arachidonic acid, eicosapentaenoic acid (EPA), and DHA in tissue lipids. An inappropriate ratio may involve too high an intake of either linoleic acid or α -linolenic acid, too little of one fatty acid, or a combination leading to an imbalance between the two series. The provision of preformed carbon chain *n*-6 and *n*-3 fatty acids results in rapid incorporation into tissue lipids. Thus, the linoleic: α -linolenic acid ratio is likely to be of most importance for diets that are very low in or devoid of arachidonic acid, EPA, and DHA. The importance of the dietary linoleic: α -linolenic acid ratio for diets rich in arachidonic acid, EPA, and DHA is not known. Arachidonic acid is important for normal growth in rats (Mohrhauer and Holman, 1963). Later in life, risk of certain diseases may be altered by arachidonic acid and arachidonic acid-derived eicosanoids. Consequently, the desirable range of *n*-6:*n*-3 fatty acids may differ with life stage.

The regulation of *n*-6 and *n*-3 fatty acid metabolism is complex as the conversion of linoleic acid to arachidonic acid is inhibited by EPA and

DHA in humans, as well as arachidonic acid, α -linolenic acid, and linoleic acid itself (Chen and Nilsson, 1993; Emken et al., 1994, 1998, 1999; Sauerwald et al., 1996). Similarly, stable isotope studies have shown that increased intakes of α -linolenic acid result in decreased conversion of linoleic acid to its metabolites, and the amounts metabolized to longer-chain metabolites is inversely related to the amount oxidized (Vermunt et al., 2000). Unfortunately, very few studies are available on the rates of formation of arachidonic acid and DHA from their precursors in humans fed diets differing in linoleic acid and α -linolenic acid content, and with or without controlled amounts of arachidonic acid, EPA, and DHA.

Arachidonic acid is a precursor to a number of eicosanoids (e.g., thromboxane A_2 , prostacyclin, and leukotriene B_4). These eicosanoids have been shown to have beneficial and adverse effects in the onset of platelet aggregation, hemodynamics, and coronary vascular tone. EPA has been shown to compete with the biosynthesis of *n*-6 eicosanoids and is the precursor of several *n*-3 eicosanoids (e.g., thromboxane A_3 , prostaglandin I_3 , and leukotriene B_5), resulting in a less thrombotic and atherogenic state (Kinsella et al., 1990).

n-6:n-3 Polyunsaturated Fatty Acid Ratio

Jensen and coworkers (1997) reported that infants fed formulas containing a linoleic acid: α -linolenic acid ratio of 4.8:1 had lower arachidonic acid concentrations and impaired growth compared to infants fed formulas containing ratios of 9.7:1 or higher. More recent, large clinical trials with infants fed formulas providing linoleic acid: α -linolenic acid ratios of 5:1 to 10:1 found no evidence of reduced growth or other problems that could be attributed to decreased arachidonic acid concentrations (Auestad et al., 1997, 2001; Makrides et al., 2000a). Clark and coworkers (1992) concluded that intake ratios less than 4:1 were likely to result in fatty acid profiles markedly different from those from infants fed human milk. Based on the limited studies, the linoleic acid: α -linolenic acid or total *n*-3:*n*-6 fatty acids ratios of 5:1 to 10:1, 5:1 to 15:1, and 6:1 to 16:1 have been recommended for infant formulas (Aggett et al., 1991; ISSFAL, 1994; LSRO, 1998).

In adult rats it has been determined that a linoleic acid: α -linolenic acid ratio of 8:1 was optimal in maintaining normal-tissue fatty acid concentrations (Bourre et al., 1996). Increasing the intake of linoleic acid from 15 to 30 g/d, with an increase in the linoleic: α -linolenic acid ratio from 8:1 to 30:1, resulted in a 40 to 54 percent decreased conversion of linoleic acid and α -linolenic acid to their metabolites in healthy men (Emken et al., 1994). Clinical studies with patients supported by total parenteral nutrition found resolution of signs of deficiency when a

parenteral lipid containing a linoleic acid: α -linolenic acid ratio of 6:1 was provided (Holman et al., 1982).

Clinical and epidemiological studies have addressed the n -6: n -3 fatty acid ratio, focusing on beneficial effects on risk of certain diseases associated with higher intakes of the n -3 fatty acids EPA and DHA, as reviewed in Chapter 11. The specific importance of the ratio in these studies cannot be assessed because the decreased ratio is secondary to an increased intake of fish or EPA and DHA from supplements. For example, low rates of heart disease in Japan, compared with the United States, have been attributed in part to a total n -6: n -3 fatty acid ratio of 4:1 (Lands et al., 1990), with about 5 percent energy as linoleic acid, 0.6 percent energy from α -linolenic acid, and 2 percent energy from EPA+DHA in Japan, compared with intakes of 6 percent energy from linoleic acid, 0.7 percent energy from α -linolenic acid, and less than 0.1 percent energy from EPA+DHA in the United States (Lands et al., 1992). Similarly, an inverse association between the dietary total n -6: n -3 fatty acid ratio and cardiovascular disease, cancer, and all-cause mortality (Dolecek and Grandits, 1991), as well as between fish intake and coronary heart disease mortality (Kromhout et al., 1985; Shekelle et al., 1985), have been reported. In other studies, however, no differences were found in coronary heart disease risk factors when a diet containing a total n -6: n -3 ratio of 4:1 compared to 1:1 was consumed (Ezaki et al., 1999), or in thrombotic conditions with a diet containing a total n -6: n -3 ratio of 3.3:1 compared with 10:1 (Nelson et al., 1991). Hu and coworkers (1999b) observed a weak relationship between the n -6: n -3 ratio and fatal ischemic heart disease since both α -linolenic acid and linoleic acid were inversely related to risk. Based on the limited studies in animals, children, and adults, a reasonable linoleic: α -linolenic acid ratio of 5:1 to 10:1 has been recommended for adults (FAO/WHO, 1994).

Impact of Trans Fatty Acids on n -6 and n -3 Metabolism

The *trans* isomers of oleic acid and linoleic acid, which are present in hydrogenated vegetable oils and meats, have been suggested to have adverse effects on growth and development through inhibition of the desaturation of linoleic acid and α -linolenic acid to arachidonic acid and DHA, respectively (Sugano and Ikeda, 1996). Desaturation and elongation of *trans* linoleic and α -linolenic acid isomers containing a double bond at the *cis*-12 and *cis*-15 position, respectively, with formation of 20 and 22 carbon chain metabolites that could be incorporated into membrane lipids, have also been suggested. In vitro studies and studies with animals fed diets high in *trans* fatty acids have found evidence of reduced essential n -6 and n -3 fatty acid desaturation (Cook, 1981; Rosenthal and Doloresco, 1984). An inverse association between total *trans* fatty acids and arachidonic

acid and DHA concentrations in plasma cholesteryl esters, and between plasma cholesteryl esters, elaidic acid (18:1 *trans*), and birth weight of premature infants has been reported (Koletzko, 1992). Studies in term infants found no relation between *trans* fatty acids and length of gestation, birth weight, or birth length (Elias and Innis, 2001). Similarly, an inverse association between plasma phospholipid *trans* fatty acids and arachidonic acid has been found for children aged 1 to 15 years (Decsi and Koletzko, 1995). The industrial hydrogenation of vegetable oils results in destruction of *cis* essential *n*-6 and *n*-3 fatty acids and the formation of *trans* fatty acids (Valenzuela and Morgado, 1999). It is not clear if differences in dietary intakes of *n*-6 and *n*-3 fatty acids, rather than inhibition of linoleic acid and α -linolenic acid desaturation by *trans* fatty acids, explains the statistical inverse associations between *trans* and *n*-6 and *n*-3 fatty acids reported in some studies (Craig-Schmidt, 2001). Based on the much greater affinity of the $\Delta 6$ desaturase for *cis* *n*-6 and *n*-3 fatty acids than monounsaturated fatty acids (Brenner, 1974; Castuma et al., 1977), and on experimental work that shows that inhibition of the $\Delta 6$ desaturation of linoleic acid is not of concern with linoleic acid intakes above about 2 percent of energy (Zevenbergen et al., 1988), it seems unlikely that inhibition of essential fatty acid metabolism by *trans* fatty acids is of concern for practical human diets.

FINDINGS BY LIFE STAGE AND GENDER GROUP

Total Fat *Infants Ages 0 Through 12 Months*

Method Used to Set the Adequate Intake

No functional criteria of fat have been demonstrated that reflects a response to dietary intake in infants. Thus, the recommended intakes of total fat are based on an Adequate Intake (AI) that reflects the observed mean fat intake of infants principally fed human milk.

Ages 0 Through 6 Months. Fat is the major single source of energy in the diet of infants exclusively fed human milk. The high intake of fat and the energy density that it provides to the diet are important in providing the energy needed for rapid growth during early infancy. Thus, the recommended intake of total fat for infants 0 through 6 months of age is based on an AI that reflects the observed mean fat intake of infants fed human milk. Table 8-2 shows the concentration and proportion of energy from fat provided by mature human milk from women delivering at term gestation. Assuming an intake of 0.78 L/d of human milk by infants exclusively fed

human milk (Chapter 1) and a mean milk fat content of 40 g/L, the AI for fat is 31 g/d. This AI assumes that the energy requirements of the young infant are being met. The mean energy content of mature human milk is 650 kcal/L (Chapter 5), thus dietary fat represents 55 percent of total energy intake for infants 0 through 6 months of age. Fomon and coworkers (1976) reported that the length and weight of infants were not different when fed formula and strained food providing 29 or 57 percent of energy from fat. Thus, an intake of 55 percent energy most likely exceeds the minimum percent needed for optimal growth of healthy infants.

Ages 7 Through 12 Months. The proportion of energy from dietary fat decreases during the second 6 months of age when complementary foods, specifically infant cereals, vegetables, and fruits, are added to the diet of the infant. The average concentration of fat in milk is approximately 40 g/L during the second 6 months of lactation (Table 8-2). The infant consumes about 0.6 L/d of human milk during the second 6 months (Chapter 1), with additional energy and nutrients provided by complementary foods, thus achieving total energy and essential nutrient needs of the infant 7 through 12 months of age.

The AI for the older infants is set based on the average intake of fat ingested from human milk and complementary foods (Chapter 1). Data from the Continuing Survey of Food Intakes by Individuals (CFSII) indicate that the average intake of fat from complementary foods by older infants is approximately 5.7 g/d. Therefore, the average fat intake from human milk and complementary foods would be 30 g/d ($[0.6 \text{ L/d} \times 40 \text{ g/L}] + 5.7$) after rounding. The average energy intake from human milk is 390 kcal/d ($0.6 \text{ L/d} \times 650 \text{ kcal/L}$) and from complementary foods is 281 kcal/d (CFSII), or a total energy intake of 671 kcal/d. Therefore, for infants 7 through 12 months of age, 40 percent of energy from fat is consumed from human milk and complementary foods.

Total Fat AI Summary, Ages 0 Through 12 Months

AI for Infants

0–6 months	31 g/d of fat
7–12 months	30 g/d of fat

Special Considerations

Conventional milk-based infant formulas contain approximately 48 percent of energy intake as fat (LSRO, 1998). The most common sources of fat in infant formulas are soybean oil, safflower oil, sunflower oil, coconut oil, and palm oil.

TABLE 8-2 Total Fat Content in Term Human Milk of Women in the United States and Canada

Reference	Study Population/ Stage of Lactation ^a	Total Fat Content (g/L)	Total Fat Content (% of total energy)	Total Energy ^b (kcal/L)
Anderson et al., 1983	9 women			
	3 d pp	18 ± 6	31.3	510 ± 90
	7 d pp	31 ± 10	43.6	630 ± 98
	14 d pp	37 ± 10	49.0	670 ± 100
Bitman et al., 1983	8-41 women			
	3 d pp	20.4 ± 3.2		
	7 d pp	28.9 ± 3.1		
	21 d pp	34.5 ± 3.7		
	42 d pp	31.9 ± 4.3		
	84 d pp	48.7 ± 6.2		
Dewey and Lönnerdal, 1983	13-18 women			
	1 mo pp	49.2 ± 10.5	55.9	781 ± 100
	2 mo pp	45.8 ± 9.7	54.0	753 ± 92
	3 mo pp	45.8 ± 16.5	55.2	736 ± 148
	4 mo pp	46.2 ± 18.6	52.1	787 ± 173
	5 mo pp	43.6 ± 16.7	51.8	747 ± 148
	6 mo pp	43.0 ± 19.6	51.0	748 ± 183
Butte et al., 1984	45 women			
	1 mo pp		47.8	
	2 mo pp		47.8	
	3 mo pp		45.7	
	4 mo pp		47.6	
Dewey et al., 1984	119 samples			
	4-6 mo pp	44.1 ± 18.5	60.2 ^c	
	7-11 mo pp	34.5 ± 15.3	47.1 ^c	
	12-20 mo pp	48.4 ± 1.19	66.0 ^c	
Ferris et al., 1988	12 women			
	2 wk pp	39.8 ± 9.9	45.2	781 ± 125
	6 wk pp	44.1 ± 11.7	51.9	753 ± 77
	12 wk pp	48.7 ± 11.9	54.5	792 ± 93
	16 wk pp	55.0 ± 10.9	58.8	829 ± 122
Innis and Kuhnlein, 1988	12 Vancouver women	31 ± 3		

TABLE 8-2 Continued

Reference	Study Population/ Stage of Lactation ^a	Total Fat Content (g/L)	Total Fat Content (% of total energy)	Total Energy ^b (kcal/L)
Nommsen et al., 1991	46–70 women			
	3 mo pp	36.2 ± 7.0	46.1	697 ± 67
	6 mo pp	37.7 ± 9.6	47.3	707 ± 92
	9 mo pp	38.1 ± 8.0	47.7	709 ± 74
	12 mo pp	37.0 ± 11.3	46.7	706 ± 110
Chen et al., 1995a	198 samples 3–4 wk pp	31.58 ± 9.37		

^a pp = postpartum.

^b Calculated using 8.87 kcal/g of fat.

^c Percent of energy determined from mean energy content of all milk samples during 7–20 mo pp (650 kcal/L).

Children and Adolescents Ages 1 Through 18 Years

A number of studies have been conducted to ascertain whether a certain amount of fat is needed in the diet to provide normal growth in children. These data generally conclude that there is no effect of fat intake on growth when consumed at levels as low as 21 percent of energy and provided that the energy intake is adequate (Boulton and Magarey, 1995; Fomon et al., 1976; Lagström et al., 1999; Lapinleimu et al., 1995; Niinikoski et al., 1997a, 1997b; Obarzanek et al., 1997; Shea et al., 1993) (see Chapter 11). There is insufficient evidence to identify a defined intake level of fat to prevent obesity or chronic diseases. Based on this lack of evidence and the lack of an effect of fat intake on growth, neither an AI nor an Estimated Average Requirement (EAR) and Recommended Dietary Allowance (RDA) are set for children and adolescents.

Adults Ages 19 Years and Older

The amount of total energy as fat in the diet can vary from 10 to 50 percent without differing effects on short-term health (Jéquier, 1999). When men and women were fed isocaloric diets containing 20, 40, or 60 percent fat, there was no difference in total daily energy expenditure (Hill et al., 1991). Similar observations were reported for individuals who consumed diets containing 10, 40, or 70 percent fat (Leibel et al., 1992) and men fed 9 to 79 percent fat (Shetty et al., 1994). In addition, a number

of studies have reported on the impact of or the relationship between low and high fat diets and the indicators for and risk of chronic diseases (e.g., coronary heart disease, diabetes, and obesity) (see Chapter 11). There are insufficient data, however, to identify a defined intake level for fat based on maintaining fat balance or on the prevention of chronic diseases. Therefore, neither an AI nor an EAR and RDA are set.

Saturated Fatty Acids

There is no evidence to indicate that saturated fatty acids are essential in the diet or have a beneficial role in the prevention of chronic diseases. Therefore, neither an AI nor an EAR and RDA are set.

cis n-9 Monounsaturated Fatty Acids

There is no evidence to indicate that monounsaturated fatty acids are essential in the diet, and monounsaturated fatty acids have no known independent role in preventing chronic diseases. Therefore, neither an AI nor an EAR and RDA are set.

n-6 Polyunsaturated Fatty Acids Infants Ages 0 Through 12 Months

Method Used to Set the AI

A series of papers reported skin lesions and poor growth in infants fed skimmed cow milk, which is very low in *n*-6 fatty acids (Hansen et al., 1958, 1963). Cuthbertson (1976) concluded that less than 50 mg/100 kcal of linoleic acid (0.45 percent energy) can provide normal health and well-being during infancy. Studies on the essential fatty acid status of older individuals have established that about 2 percent energy from *n*-6 polyunsaturated fatty acids (linoleic acid) will prevent abnormal elevation of the triene:tetraene ratio (20:3*n*-9:20:4*n*-6) and clinical signs of essential fatty acid deficiency during parenteral nutrition (Barr et al., 1981). Interpretation, however, is complicated because linoleic acid in the soybean oil emulsion used to provide *n*-6 fatty acids can also be expected to inhibit synthesis of eicosatrienoic acid (20:3*n*-9) (Brenner, 1974), and thus reduce the triene:tetraene ratio. Furthermore, children are expected to require higher amounts of *n*-6 fatty acids than adults in order to support deposition of *n*-6 fatty acids in cell membranes of growing tissues. This suggests that a margin of safety is prudent.

Ages 0 Through 6 Months. An AI can be set based on the average amount of *n*-6 polyunsaturated fatty acids provided by human milk. Table 8-2 provides the fat and energy content of human milk. Human milk contains 5.6 g/L (14 percent *n*-6 fatty acid in milk $\times$ 40 g/L) of *n*-6 polyunsaturated fatty acids (Table 8-3).

Based on an average intake of 0.78 L/d of human milk (Chapter 1), the AI is 4.4 g/d ($0.78 \text{ L/d} \times 5.6 \text{ g/L}$). The energy content of human milk is approximately 650 kcal/L (Chapter 5) and therefore provides 507 kcal/d ($650 \text{ kcal/L} \times 0.78 \text{ L/d}$). Thus, *n*-6 polyunsaturated fatty acids contribute approximately 8 percent of daily energy intake. The various *n*-6 fatty acids that are naturally present in human milk can contribute to this AI.

Ages 7 Through 12 Months. The period from 7 through 12 months of age is a time of major transition in the diet, from infants exclusively fed human milk or infant formulas that provide large amounts of dietary fat to a diet containing a variety of foods in addition to milk or formula. The infant consumes about 0.6 L/d of human milk during the second 6 months of life (Chapter 1), with additional energy and nutrients provided by complementary foods, thus achieving total energy and essential nutrient needs. The AI for older infants is set based on the average intake of *n*-6 polyunsaturated fatty acids ingested from human milk and complementary foods (Chapter 1). Data from CFSII indicates that the average intake of *n*-6 polyunsaturated fatty acids from complementary foods by older infants is approximately 1.2 g/d. Therefore, the AI for *n*-6 polyunsaturated fatty acids is 4.6 g/d ($[0.6 \text{ L/d} \times 5.6 \text{ g/L}] + 1.2$) after rounding. The average fat energy coming from human milk is 390 kcal/d ($0.6 \text{ L/d} \times 650 \text{ kcal/L}$), and from complementary foods is 281 kcal/d (CFSII), for a total energy intake of 671 kcal/d. Therefore, 6 percent of energy from *n*-6 polyunsaturated fat is consumed via human milk and complementary foods.

n-6 Polyunsaturated Fatty Acids AI Summary,
Ages 0 Through 12 Months

AI for Infants

0–6 months	4.4 g/d of <i>n</i>-6 polyunsaturated fatty acids
7–12 months	4.6 g/d of <i>n</i>-6 polyunsaturated fatty acids

Special Considerations

The polyunsaturated vegetables oils (e.g., safflower oil and soybean oil) used in the manufacture of infant formulas contain abundant amounts (45 to 70 percent of total fatty acids) of linoleic acid. The minimum permissible amount of linoleic acid found in infant formulas is 2.7 percent of

TABLE 8-3 *n*-6 Polyunsaturated Fatty Acid Content in Term Human Milk of Women in the United States and Canada

Reference	<i>n</i>	<i>n</i> -6 Fatty Acid	Content in Human Milk	
			% of Total Fatty Acids	% of Total Energy ^a
Putnam et al., 1982	9	18:2	15.8 ± 0.61	8.62
		20:2	0.4 ± 0.03	0.22
		20:3	0.4 ± 0.03	0.22
		20:4	0.6 ± 0.03	0.33
		22:4	0.2 ± 0.02	0.11
		22:5	0.1 ± 0.02	0.05
		Total	17.50	9.55
Bitman et al., 1983	6	18:2	15.58 ± 1.99	8.50
		20:2	0.18 ± 0.20	0.10
		20:3	0.53 ± 0.15	0.29
		20:4	0.60 ± 0.29	0.33
		22:4	0.07 ± 0.16	0.04
		22:5	0.03 ± 0.08	0.02
		Total	16.99	9.28
Harris et al., 1984	8	18:2	15.3 ± 3.3	8.35
		20:3	0.3 ± 0.1	0.16
		20:4	0.4 ± 0.1	0.22
		Total	16.0	8.73
Finley et al., 1985	172	18:2	16.49 ± 4.80	9.00
		20:2	0.38 ± 0.15	0.21
		20:3	0.28 ± 0.09	0.15
		20:4	0.29 ± 0.08	0.16
		Total	17.44	9.52
Innis and Kuhnlein, 1988	12	18:2	12.7 ± 1.8	6.93
		20:2	0.4 ± 0.1	0.22
		20:4	0.7 ± 0.0	0.38
		22:5	0.2 ± 0.1	0.11
		Total	14.0	7.64
Chen et al., 1995a	198	18:2	10.47 ± 2.62	5.72
		18:3	0.08 ± 0.06	0.04
		20:2	0.17 ± 0.37	0.09
		20:3	0.26 ± 0.09	0.14
		20:4	0.35 ± 0.11	0.19
		22:4	0.04 ± 0.05	0.02
		22:5	0.01 ± 0.02	0.01
		Total	11.38	6.21

TABLE 8-3 Continued

Reference	<i>n</i>	<i>n</i> -6 Fatty Acid	Content in Human Milk	
			% of Total Fatty Acids	% of Total Energy ^a
Innis and King, 1999	103	18:2	12.1 ± 0.35	6.60
		18:3	0.1 ± 0.00	0.05
		20:2	0.3 ± 0.01	0.16
		20:3	0.3 ± 0.01	0.16
		20:4	0.4 ± 0.01	0.22
		22:4	0.1 ± 0.00	0.05
		Total	13.3	7.24

^a Calculated using the following values: 40 g of fat/L of milk, 8.87 kcal/g of fat, 650 kcal/L of milk.

energy (Infant Formula. Nutrient Specifications. 21 C.F.R. §107.100, 1985); however, formulas provide higher amounts than this level.

Children and Adolescents Ages 1 Through 18 Years

Method Used to Set the AI

No specific information is available on the amount of linoleic acid required to correct the symptoms of an *n*-6 polyunsaturated fatty acid deficiency. In the absence of this information, an AI is set based on the median intake of linoleic acid consumed in the United States where the presence of an *n*-6 fatty acid deficiency is basically nonexistent in the free-living population (Appendix Table E-9), and rounding.

Linoleic Acid AI Summary, Ages 1 Through 18 Years

AI for Children

1–3 years 7 g/d of linoleic acid
4–8 years 10 g/d of linoleic acid

AI for Boys

9–13 years 12 g/d of linoleic acid
14–18 years 16 g/d of linoleic acid

AI for Girls

9–13 years	10 g/d of linoleic acid
14–18 years	11 g/d of linoleic acid

*Adults Ages 19 Years and Older**Method Used to Set the AI*

Various studies on adult patients receiving total parenteral nutrition have shown that linoleic acid intakes of as little as 7.4 to 8 g/d reverses the symptoms of deficiency (Barr et al., 1981; Collins et al., 1971; Goodgame et al., 1978; Jeppesen et al., 1998; Wong and Deitel, 1981). There is inadequate information, however, to set an EAR for healthy individuals. In the absence of this information, an AI is set based on the median intake of linoleic acid in the United States where the presence of an *n*-6 fatty acid deficiency is basically nonexistent in the free-living population (Appendix Table E-9). The highest median intakes have been used, each for men and women 19 to 50 years of age. Energy expenditure increases fat oxidation (Calles-Escandon et al., 1996) and linoleic acid is readily used for energy (Cunnane et al., 2001). Therefore, the AI for older men and women (greater than 50 years of age), whose energy expenditure is less than younger adults, is based on the highest median intake within this age range and rounding.

*Linoleic Acid AI Summary, Ages 19 Years and Older***AI for Men**

19–30 years	17 g/d of linoleic acid
31–50 years	17 g/d of linoleic acid
51–70 years	14 g/d of linoleic acid
> 70 years	14 g/d of linoleic acid

AI for Women

19–30 years	12 g/d of linoleic acid
31–50 years	12 g/d of linoleic acid
51–70 years	11 g/d of linoleic acid
> 70 years	11 g/d of linoleic acid

Pregnancy

Method Used to Set the AI

The demand for *n*-6 fatty acids for incorporation into placental tissue and the developing fetus during gestation must be met by *n*-6 fatty acids from maternal tissues or through dietary intake. Longitudinal studies have reported a decrease in plasma arachidonic acid concentration in pregnant women (Ghebremeskel et al., 2000; Sanjurjo et al., 1993). Lower arachidonic acid concentrations have also been reported for red blood cell phospholipids of pregnant women compared with nonpregnant women (Ghebremeskel et al., 2000). It is not clear that this reflects an increased need for *n*-6 fatty acids that was not met in the women in these studies, or whether changes in maternal *n*-6 fatty acid concentrations are normal physiological responses explained by the changes in endocrine status, lipoprotein and lipid metabolism, or nutrient transfer to the fetus. There is no evidence that maternal dietary intervention with *n*-6 fatty acids has any effect on fetal or infant growth and development in women meeting the requirements for *n*-6 fatty acids.

Because of a lack of evidence for determining the requirement during pregnancy, the AI is set based on the median linoleic acid intake of pregnant women in the United States where a deficiency is basically nonexistent in noninstitutionalized populations (Appendix Table E-9), and rounding.

Linoleic Acid AI Summary, Pregnancy

AI for Pregnant Women

14–18 years	13 g/d of linoleic acid
19–30 years	13 g/d of linoleic acid
31–50 years	13 g/d of linoleic acid

Lactation

Method Used to Set the AI

As stated above, there is no evidence that maternal dietary intervention with *n*-6 fatty acids has any effect on infant growth and development in women meeting the requirements for *n*-6 fatty acids. Because of a lack of evidence for determining the requirement during lactation, the AI is set based on the median linoleic acid intake of lactating women in the United States where a deficiency is basically nonexistent in noninstitutionalized populations (Appendix Table E-9), and rounding.

*Linoleic Acid AI Summary, Lactation***AI for Lactation**

14–18 years	13 g/d of linoleic acid
19–30 years	13 g/d of linoleic acid
31–50 years	13 g/d of linoleic acid

*n-3 Polyunsaturated Fatty Acids
Infants Ages 0 Through 12 Months**Method Used to Set the AI*

Human milk contains α -linolenic acid (18:3), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6) (Table 8-4), but the amounts present are highly variable and depend on the amounts present in the mother's diet. Concentrations of about 0.7 to 1.4 percent DHA have been reported for women who eat large amounts of fish and other marine foods (Innis and Kuhnlein, 1988; Kneebone et al., 1985). Blood concentrations of DHA appear to show little metabolic regulation and increase with increasing DHA intake in breast-fed infants (Gibson et al., 1997; Innis and King, 1999; Sanders and Reddy, 1992) or formula-fed infants (Auestad et al., 1997; Carlson et al., 1996a; Innis et al., 1996; Makrides et al., 1995), as they do in adults. Numerous studies have shown that infants fed formulas with no DHA have lower plasma and red blood cell DHA concentrations than infants fed human milk or formulas with DHA (Auestad et al., 1997; Carlson et al., 1986, 1996a; Innis et al., 1996; Makrides et al., 1995; Ponder et al., 1992; Putnam et al., 1982). Similarly, the plasma and red blood cell DHA concentrations are lower in infants breast-fed by mothers with vegetarian rather than omnivorous diets (Sanders and Reddy, 1992). Evidence of DHA depletion based on functional endpoints has not been reported for populations or subgroups that have diets containing no DHA but with adequate α -linolenic acid.

Several autopsy studies have reported lower DHA concentrations in the brains of infants fed formulas that contain no DHA compared with infants fed human milk (Byard et al., 1995; Farquharson, 1994; Farquharson et al., 1992, 1995; Jamieson et al., 1994, 1999; Makrides et al., 1994). In addition, brain DHA accumulation continues in both breast-fed and formula-fed infants for at least 40 weeks of life, but the accumulation is at a greatly reduced rate in formula-fed infants (Makrides et al., 1996). Although many infant formulas contain similar amounts of α -linolenic acid as human milk, the dietary supply of only α -linolenic acid and no DHA in formulas may be inadequate to supply the infant brain with DHA (Farquharson,

TABLE 8-4 *n*-3 Polyunsaturated Fatty Acid Content in Term Human Milk of Women in the United States and Canada

Reference	<i>n</i>	<i>n</i> -3 Fatty Acid	Content in Human Milk	
			% of Total Fatty Acids	% of Total Energy ^a
Putnam et al., 1982	9	18:3	0.8 ± 0.09	0.44
		20:5	0.1 ± 0.03	0.05
		22:5	0.1 ± 0.01	0.05
		22:6	0.1 ± 0.01	0.05
		Total	1.1	0.59
Bitman et al., 1983	6	18:3	1.03 ± 0.21	0.56
		20:5	trace	trace
		22:5	0.11 ± 0.15	0.06
		22:6	0.23 ± 0.14	0.13
		Total	1.37	0.75
Harris et al., 1984	8	18:3	0.8 ± 0.5	0.44
		20:5	trace	trace
		22:5	trace	trace
		22:6	0.1 ± 0.1	0.05
		Total	0.9	0.49
Finley et al., 1985	172	18:3	1.56 ± 0.43	0.85
		22:6	0.06 ± 0.004	0.03
		Total	1.62	0.88
Innis and Kuhnlein, 1988	12	18:3	0.6 ± 0.2	0.33
		20:5	0.2 ± 0.2	0.11
		22:5	0.4 ± 0.1	0.22
		22:6	0.4 ± 0.1	0.22
		Total	1.6	0.88
Chen et al., 1995a	198	18:3	1.16 ± 0.37	0.63
		20:4	0.06 ± 0.06	0.03
		20:5	0.05 ± 0.05	0.03
		22:5	0.08 ± 0.06	0.04
		22:6	0.14 ± 0.10	0.08
		Total	1.49	0.81
Innis and King, 1999	103	18:3	1.4 ± 0.07	0.76
		20:5	0.1 ± 0.01	0.05
		22:5	0.2 ± 0.02	0.11
		22:6	0.2 ± 0.03	0.11
		Total	1.9	1.03

^a Calculated using the following values: 40 g of fat/L of milk, 8.87 kcal/g of fat, 650 kcal/L of milk.

1994). Animal studies have shown that dietary DHA is incorporated into brain tissue to a greater extent than is DHA that is biosynthesized from α -linolenic acid (Abedin et al., 1999; Sinclair, 1975). Furthermore, administration of dietary α -linolenic acid was not effective in restoring brain DHA concentrations in chicks deficient in n -3 fatty acids (Anderson et al., 1990). Therefore, the DHA content of the brain may depend more heavily upon the dietary supply of DHA rather than its precursor, α -linolenic acid. Randomized clinical studies on growth or neural development with term infants fed formulas currently yield conflicting results on the requirement for n -3 fatty acids in young infants (see "Evidence Considered for Estimating the Requirement for Total Fat and Fatty Acids").

Ages 0 Through 6 Months. n -3 Polyunsaturated fatty acids provide DHA that is important for the developing brain and retina. Human milk is assumed to meet the n -3 fatty acid requirements of the infants fed human milk. Therefore, an AI for n -3 fatty acids is based on the amount of n -3 fatty acids, total fat, and energy provided by human milk. Table 8-2 shows the fat and energy content of human milk. Human milk contains approximately 0.63 g/L (1.58 percent n -3 fatty acids $\times$ 40 g/L total fat) of n -3 polyunsaturated fatty acids (Table 8-4). The AI is based on the average amount of milk consumed by the infant (0.78 L/d) and the n -3 fatty acid concentration in human milk. Therefore, the AI is set at 0.5 g/d (0.78 L/d $\times$ 0.63 g/L), after rounding, which provides approximately 4.5 kcal/d. Because human milk provides 650 kcal/L (Chapter 5) or 507 kcal/d (650 kcal/L $\times$ 0.78 L/d), an AI of 0.5 g/d of n -3 polyunsaturated fatty acids represents approximately 1 percent (4.5 $\div$ 507) energy intake, after rounding. The various n -3 fatty acids that are naturally present in human milk can contribute to this AI.

Ages 7 Through 12 Months. While the energy requirement relative to body weight decreases in the second 6 months of life (see Chapter 5), autopsy analyses suggest that brain DHA accretion continues at a similar rate from 0 through 24 months of age (Martinez, 1992). The AI for older infants is set based on the average intake of n -3 fatty acids ingested from human milk and complementary foods (Chapter 1). Data from CFSII indicate that the average intake of n -3 fatty acids from complementary foods by older infants is approximately 0.11 g/d. Therefore, the AI is 0.5 g/d [0.6 L/d $\times$ 0.63 g/L] + 0.11), after rounding, which represents approximately 4.5 kcal/d. The average energy intake from human milk is 390 kcal/d (0.6 L/d $\times$ 650 kcal/L), and from complementary foods is 281 kcal/d (CFSII), for a total energy intake of 671 kcal/d. Therefore, approximately 0.67 percent (4.5 kcal/d $\div$ 671 kcal/d) of energy is consumed as n -3 polyunsaturated fatty acids from human milk and complementary foods.

*n-3 Polyunsaturated Fatty Acid AI Summary, Ages 0 Through 12 Months***AI for Infants**

0–6 months	0.50 g/d of <i>n</i>-3 polyunsaturated fatty acids
7–12 months	0.50 g/d of <i>n</i>-3 polyunsaturated fatty acids

Special Considerations

Vegetable oils that provide α -linolenic acid are used in the manufacture of infant formulas. The U.S. Code of Federal Regulations does not currently specify minimum or maximum levels of α -linolenic acid for infant formulas. At the present time, DHA is not directly added to infant formulas. Information from clinical trials with term infants fed formulas with DHA are inconsistent, and associations between lower growth and delays on some developmental tests have been noted in preterm and term infants fed formulas containing DHA, but not arachidonic acid. Definitive evidence that this is due to the absence of arachidonic acid or explained by antagonism between DHA and *n*-6 fatty acids is not available. DHA is added to infant formula ingredients in the form of oils from fish oils, egg total lipids, egg phospholipids, and oil from single cell microorganisms.

*Children and Adolescents Ages 1 Through 18 Years**Method Used to Set the AI*

One case study of a 6-year-old girl on total parenteral nutrition (TPN) reported that the TPN solution, which was low in α -linolenic acid and provided approximately 0.08 g/d, resulted in episodes of numbness, weakness, blurred vision, and the inability to walk (Holman et al., 1982). Analysis of the girl's plasma fatty acids confirmed a low *n*-3 fatty acid concentration. It was determined that 1.625 g/d of α -linolenic acid reversed the abnormal neurological symptoms. Bjerve and coworkers (1988) reported low plasma *n*-3 fatty acid concentrations and poor growth in a child fed approximately 0.54 g/d of α -linolenic acid via a gastric tube. Growth was somewhat improved by the addition of 0.56 g/d of α -linolenic acid.

Because of a lack of evidence for determining the requirement for *n*-3 fatty acids during childhood, an AI is set based on the median intake of α -linolenic acid in the United States where a deficiency is basically nonexistent in noninstitutionalized populations (Appendix Table E-11), and rounding. Small amounts of EPA and DHA can contribute toward reversing an *n*-3 fatty acid deficiency (Bjerve, 1989; Bjerve et al., 1987a, 1987b, 1989) and can therefore contribute toward the AI for α -linolenic acid.

EPA and DHA contribute approximately 10 percent of the total *n*-3 fatty acid intake and therefore this percent contributes toward the AI for α -linolenic acid (Appendix Tables E-10, E-12, and E-14).

α -Linolenic Acid AI Summary, Ages 1 Through 18 Years

AI for Children

1–3 years	0.7 g/d of α -linolenic acid
4–8 years	0.9 g/d of α -linolenic acid

AI for Boys

9–13 years	1.2 g/d of α -linolenic acid
14–18 years	1.6 g/d of α -linolenic acid

AI for Girls

9–13 years	1.0 g/d of α -linolenic acid
14–18 years	1.1 g/d of α -linolenic acid

Adults Ages 19 Years and Older

Method Used to Set the AI

Several studies involving adult patients who were fed by gastric tube showed that an *n*-3 fatty acid (α -linolenic acid) deficiency could occur with intakes ranging from 0.015 to 0.095 g/d of α -linolenic acid (Bjerve, 1989; Bjerve et al., 1987a, 1987b, 1989), whereas intakes of as low as 0.3 g/d prevented the symptoms of a deficiency (Bjerve et al., 1987a). There were insufficient data, however, to set an EAR for free-living healthy adults.

Because of a lack of evidence for determining the requirement for *n*-3 fatty acids, an AI is set based on the highest median intake of α -linolenic acid by adults in the United States where a deficiency is basically non-existent in noninstitutionalized populations (Appendix Table E-11), and rounding. Small amounts of EPA and DHA can contribute toward reversing an *n*-3 fatty acid deficiency (Bjerve, 1989; Bjerve et al., 1987a, 1987b, 1989). EPA and DHA contribute approximately 10 percent of the total *n*-3 fatty acid intake and therefore this percent contributes toward the AI for α -linolenic acid (Appendix Tables E-10, E-12, and E-14).

*α -Linolenic Acid AI Summary, Ages 19 Years and Older***AI for Men**

19–30 years	1.6 g/d of α -linolenic acid
31–50 years	1.6 g/d of α -linolenic acid
51–70 years	1.6 g/d of α -linolenic acid
> 70 years	1.6 g/d of α -linolenic acid

AI for Women

19–30 years	1.1 g/d of α -linolenic acid
31–50 years	1.1 g/d of α -linolenic acid
51–70 years	1.1 g/d of α -linolenic acid
> 70 years	1.1 g/d of α -linolenic acid

*Pregnancy and Lactation**Method Used to Set the AI*

The demand for *n*-3 polyunsaturated fatty acids for incorporation into placental tissue and for the developing fetus during gestation, as well as for secretion of *n*-3 polyunsaturated fatty acids in milk during lactation, must be met by *n*-3 fatty acids from maternal tissues or through dietary intake. Several studies have reported lower plasma and red blood cell lipid DHA concentrations in pregnant and lactating women compared with non-pregnant, nonlactating women (Ghebremeskel et al., 2000; Holman et al., 1991). It is not clear that this reflects declining DHA status due to inadequate *n*-3 fatty acid intakes in the women in these studies. An alternative explanation is that changes in maternal DHA concentrations are normal physiological responses to the changes in endocrine status, lipoprotein and lipid metabolism, or nutrient transfer that accompany pregnancy and lactation. However, supplementation with fish oil during pregnancy does increase DHA in both the mother and the newborn infant, and supplementation with fish oil during lactation increases the concentration of DHA in the mother's milk and in the infant's blood (Connor et al., 1996; Henderson et al., 1992; van Houwelingen et al., 1995). Dietary fatty acids are almost completely absorbed, and an increase in blood DHA concentration following the increase in intake with fish oil supplementation is to be expected. Evidence is not available to show that increasing intakes of DHA in pregnant and lactating women consuming diets that meet requirements for *n*-6 and *n*-3 fatty acids have any physiologically significant benefit to the infant. Population comparative studies have found higher birthweights and longer gestation for women in the Faroe Islands than in Denmark (Olsen et al., 1989). This has been attributed to a higher intake of EPA from fish

and other marine foods, leading to *n*-3 fatty acid-induced inhibition of the *n*-6 fatty acid-derived eicosanoids that are important in cervical ripening and initiation of parturition. Subsequent intervention studies indicate that 10.8 g of supplemental *n*-3 fatty acids from fish oil is associated with an increase in gestation of about 4 days (Olsen et al., 1992).

Because of a lack of evidence for determining the requirement for *n*-3 fatty acids during pregnancy and lactation, an AI is set based on the median intake of α -linolenic acid in the United States where a deficiency is basically nonexistent in noninstitutionalized populations (Appendix Table E-11), and rounding. Small amounts of EPA and DHA can contribute toward reversing an *n*-3 fatty acid deficiency (Bjerve, 1989; Bjerve et al., 1987a, 1987b, 1989), and can therefore contribute toward the AI for α -linolenic acid.

α -Linolenic Acid AI Summary, Pregnancy and Lactation

AI for Pregnancy

14–18 years	1.4 g/d of α-linolenic acid
19–30 years	1.4 g/d of α-linolenic acid
31–50 years	1.4 g/d of α-linolenic acid

AI for Lactation

14–18 years	1.3 g/d of α-linolenic acid
19–30 years	1.3 g/d of α-linolenic acid
31–50 years	1.3 g/d of α-linolenic acid

Special Considerations

The ratio of linoleic acid: α -linolenic acid in the diet is important because linoleic acid and α -linolenic acid compete for the same desaturase enzymes. Thus, a high ratio of linoleic acid: α -linolenic acid can inhibit the conversion of α -linolenic acid to DHA, while a low ratio will inhibit the desaturation of linoleic acid to arachidonic acid. The linoleic acid: α -linolenic acid ratio, however, is likely to be of greatest importance in diets that are very low or devoid of arachidonic acid, EPA, and DHA.

The available data, although limited, suggest that linoleic: α -linolenic acid ratios below 5:1 may be associated with impaired growth in infants (Jensen et al., 1997). Although a ratio of 30:1 has been shown to reduce further metabolism of α -linolenic acid, sufficient dose-response data are not available to set an upper range for this ratio with confidence. Assuming an intake of *n*-6 fatty acids of 5 percent energy, with this being mostly linoleic acid, the α -linolenic acid intake at a 5:1 ratio would be 1 percent of energy.

Trans Fatty Acids

There are no data available to indicate a health benefit from consuming *trans* fatty acids. Therefore, neither an AI nor an EAR and RDA are established for *trans* fatty acids.

INTAKES OF TOTAL FAT AND FATTY ACIDS

Total Fat

Food Sources

Both animal- and plant-derived food products contain fat. The principal foods that contribute to fat intake are butter, margarine, vegetable oils, visible fat on meat and poultry products, whole milk, egg yolks, nuts, and baked goods (e.g., cookies, doughnuts, and cakes). Over 95 percent of total fat intake is in the form of triacylglycerols. As discussed below, the type of fat present in these food products varies.

Dietary Intake

Intake data from the Continuing Survey of Food Intakes of Individuals (CFSII) (1994–1996, 1998) showed that the median total fat intake ranged from 65 to 100 g/d for men and 48 to 63 g/d for women (Appendix Table E-5). These intake ranges represent approximately 32 to 34 percent of total energy (Appendix Table E-6). During 1990 to 1997, median intakes of fat ranged from 32 to 34 percent and 30 to 33 percent of energy in Canadian men and women, respectively (Appendix Table F-3).

A longitudinal study in the United States found that dietary fat represented 48, 41, 35, and 30 percent of total energy intakes at 3, 6, 12, and 24 months of age, respectively (Butte, 2000). The Third National Health and Nutrition Examination Survey (NHANES) estimated that children 2 to 19 years of age consumed an average of 34 percent of total energy as fat, with little difference across the individual age groups (Troiano et al., 2000). Comparison of data collected across the three NHANES studies conducted since the early 1970s shows that children and adolescents across all race, gender, and age groups have decreased their total fat intake. Mean age-adjusted fat intakes have declined from 36 to 37 percent to 33 to 34 percent of total energy (Troiano et al., 2000). About 23 percent of children 2 to 5 years old, 16 percent of children 6 to 11 years old, and 15 percent of adolescents 12 to 19 years old had dietary fat intakes equal to or less than 30 percent of total energy intakes.

Saturated Fatty Acids

Food Sources

Sources of saturated fatty acids tend to be foods of animal sources, including whole milk, cream, butter, cheese, and fatty meats such as pork and beef (USDA/HHS, 2000). Certain oils, however, such as coconut, palm, and palm kernel oil, also contain relatively high amounts of saturated fatty acids. Saturated fatty acids provide approximately 20 to 25 percent of energy in human milk (Table 8-5).

Dietary Intake

Based on intake data from CFSII (1994–1996, 1998), median saturated fatty acid intake ranged from approximately 21 to 34 g/d for men and 15 to 21 g/d for women (Appendix Table E-7). Data from NHANES III indicated that saturated fatty acids provided 11 to 12 percent of energy in adult diets and ranged from 12.2 to 13.9 percent of energy for children and adolescents (CDC, 1994). NHANES III reported that 9 percent of children 2 to 11 years old and 7 percent of those 12 to 19 years old had saturated fatty acid intakes of less than 10 percent of total energy (Troiano et al., 2000). During 1990 to 1997, median intakes of saturated fatty acids ranged from approximately 10 to 12 percent of energy for Canadian men and women (Appendix Table F-4).

Cis-Monounsaturated Fatty Acids

Food Sources

About 50 percent of monounsaturated fatty acids are provided by animal products, primarily meat fat (Jonnalagadda et al., 1995). Oils that contain monounsaturated fatty acids include canola and olive oils. Monounsaturated fatty acids provide approximately 20 percent of energy in human milk (Table 8-6).

Dietary Intake

Based on intake data from CFSII (1994–1996, 1998), median monounsaturated fatty acid intake ranged from approximately 25 to 39 g/d for men and 18 to 24 g/d for women (Appendix Table E-8). Data from the 1987–1988 Nationwide Food Consumption Survey indicated that mean intakes of monounsaturated fatty acids were 13.6 to 14.3 percent of energy (Ganji and Betts, 1995).

TABLE 8-5 Saturated Fatty Acid Content in Term Human Milk of Women in the United States and Canada

Reference	<i>n</i>	Saturated Fatty Acid	Content in Human Milk	
			% of Total Fatty Acids	% of Total Energy ^a
Putnam et al., 1982	9	8:0	0.3	0.16
		10:0	1.4	0.76
		12:0	6.2	3.38
		14:0	7.6	4.15
		16:0	20.5 ± 0.70	11.19
		18:0	9.0 ± 0.46	4.91
		20:0	0.3 ± 0.02	0.16
		21:0	0.1 ± 0.02	0.05
		24:0	0.5 ± 0.01	0.27
		Total	45.9	25.03
Bitman et al., 1983	6	10:0	0.97 ± 0.28	0.53
		12:0	4.46 ± 1.17	2.43
		14:0	5.68 ± 1.36	3.10
		15:0	0.31 ± 0.07	0.17
		16:0	22.20 ± 2.28	12.12
		17:0	0.49 ± 0.36	0.27
		18:0	7.68 ± 1.85	4.19
		20:0	0.32 ± 0.11	0.17
		21:0	0.17 ± 0.12	0.09
		Total	42.28	23.07
Harris et al., 1984	8	10:0	trace	trace
		12:0	4.2 ± 1.3	2.29
		14:0	5.9 ± 0.7	3.22
		16:0	22.8 ± 1.6	12.45
		18:0	8.2 ± 1.2	4.48
		Total	41.1	22.44
Finley et al., 1985	172	8:0	0.16 ± 0.11	0.09
		10:0	1.10 ± 0.30	0.60
		12:0	5.56 ± 1.68	3.03
		14:0	8.01 ± 2.46	4.37
		16:0	23.28 ± 3.35	12.71
		18:0	8.06 ± 1.58	4.40
		Total	46.17	25.20
Innis and Kuhnlein, 1988	12	10:0	1.2 ± 0.2	0.66
		12:0	5.2 ± 0.7	2.84
		14:0	6.7 ± 0.5	3.66
		16:0	22.1 ± 2.7	12.06
		18:0	8.2 ± 0.8	4.48
		Total	43.4	23.70

continued

TABLE 8-5 Continued

Reference	n	Saturated Fatty Acid	Content in Human Milk	
			% of Total Fatty Acids	% of Total Energy ^a
Chen et al., 1995a	198	10:0	1.39 ± 0.59	0.76
		12:0	5.68 ± 2.01	3.10
		14:0	6.10 ± 1.73	3.33
		15:0	0.37 ± 0.12	0.20
		16:0	18.30 ± 2.25	9.99
		17:0	0.32 ± 0.08	0.17
		18:0	6.15 ± 0.97	3.36
		20:0	0.15 ± 0.09	0.08
		Total	38.46	20.99
Innis and King, 1999	103	10:0	0.6 ± 0.03	0.33
		12:0	4.1 ± 0.15	2.24
		14:0	6.1 ± 0.21	3.33
		16:0	19.4 ± 0.28	10.59
		18:0	7.2 ± 0.15	3.93
		20:0	0.2 ± 0.00	0.11
		22:0	0.1 ± 0.00	0.05
		24:0	0.1 ± 0.00	0.05
		Total	37.8	20.63

^a Calculated using the following values: 40 g of fat/L of milk, 8.87 kcal/g of fat, 650 kcal/L of milk.

n-6 Polyunsaturated Fatty Acids

Food Sources

Sources of *n-6* polyunsaturated fatty acids include nuts, seeds, certain vegetables, and vegetable oils such as soybean oil, safflower oil, and corn oil. Certain oils, such as blackcurrant seed oil and evening primrose oil, are high in γ -linolenic acid (18:3*n-6*), which is an intermediate in the conversion of linoleic acid to arachidonic acid. Arachidonic acid is formed from linoleic acid in animal cells, but not plant cells, and is present in the diet in small amounts in meat, poultry, and eggs. Arachidonic acid is not present in plant-derived fats and oils.

TABLE 8-6 Monounsaturated Fatty Acid Content in Term Human Milk of Women in the United States and Canada

Reference	n	Monounsaturated Fatty Acid	Content in Human Milk	
			% of Total Fatty Acids	% of Total Energy ^a
Putnam et al., 1982	9	18:1	37.6 ± 0.75	20.52
		20:1	0.9 ± 0.07	0.49
		22:1	0.1 ± 0.02	0.05
		Total	38.6	21.06
Bitman et al., 1983	6	16:1	3.83 ± 0.39	2.09
		18:1	35.51 ± 2.73	19.38
		Total	39.34	21.47
Harris et al., 1984	8	16:1	2.5 ± 0.6	1.36
		18:1	32.6 ± 3.3	17.79
		20:1	0.5 ± 0.1	0.27
		Total	35.6	19.42
Finley et al., 1985	172	16:1	3.02 ± 0.77	1.65
		18:1	31.72 ± 3.81	17.31
		Total	34.74	18.96
Innis and Kuhnlein, 1988	12	16:1	3.3 ± 0.6	1.80
		18:1	36.3 ± 2.7	19.81
		20:1	0.7 ± 0.3	0.38
		22:1	0.2 ± 0.1	0.11
		Total	40.5	22.10
Chen et al., 1995a	198	14:1	0.28 ± 0.08	0.15
		16:1	2.68 ± 0.69	1.46
		17:1	0.21 ± 0.06	0.11
		18:1	36.09 ± 3.51	19.70
		20:1	0.53 ± 0.22	0.29
		22:1	0.02 ± 0.03	0.01
		Total	39.81	21.72
Innis and King, 1999	103	14:1	0.2 ± 0.01	0.11
		16:1	2.5 ± 0.08	1.36
		18:1	35.7 ± 0.41	19.49
		20:1	0.6 ± 0.05	0.33
		22:1	0.2 ± 0.02	0.11
		24:1	0.1 ± 0.01	0.05
		Total	39.3	21.45

^a Calculated using the following values: 40 g of fat/L of milk, 8.87 kcal/g of fat, 650 kcal/L of milk.

Dietary Intake

Based on intake data from CFSII (1994–1996, 1998), median *n*-6 polyunsaturated fatty acid (linoleic acid) intake ranged from approximately 12 to 17 g/d for men and 9 to 11 g/d for women (Appendix Table E-9).

Polyunsaturated fatty acids have been reported to contribute approximately 5 to 7 percent of total energy intake in diets of adults (Allison et al., 1999; Fischer et al., 1985). Most (approximately 85 to 90 percent) *n*-6 polyunsaturated fatty acids are consumed in the form of linoleic acid. Other *n*-6 polyunsaturated fatty acids, such as arachidonic acid and γ -linolenic acid, are present in small amounts in the diet.

n-3 Polyunsaturated Fatty Acids

Food Sources

The major sources of *n*-3 fatty acids include certain vegetable oils and fish (Kris-Etherton et al., 2000). Vegetable oils such as soybean and flax-seed oils contain high amounts of α -linolenic acid. Fish oils provide a mixture of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and fatty fish are the major dietary sources of EPA and DHA. Smaller amounts are also present in meat and eggs.

Dietary Intake

Based on intake data from CFSII (1994–1996, 1998), the total *n*-3 fatty intake for men and women ranged from approximately 1.3 to 1.8 g/d and 1.0 to 1.2 g/d, respectively (Appendix Table E-10). These findings are similar to that reported by Kris-Etherton and coworkers (2000), who also reported that the average intake of *n*-3 polyunsaturated fatty acids was approximately 0.7 percent of energy. The median intake of α -linolenic acid ranged from approximately 1.2 to 1.6 g/d for men and 0.9 to 1.1 g/d for women (Appendix Table E-11). For all adults, the median intakes of EPA and DHA ranged from 0.004 to 0.007 and 0.052 to 0.093 g/d, respectively (Appendix Tables E-12 and E-14). The median intake of DHA ranged from 0.066 to 0.093 g/d for men and 0.052 to 0.069 g/d for women (Appendix Table E-14). Docosapentaenoic acid provided only 0.001 to 0.005 g/d (Appendix Table E-13).

Trans Fatty Acids

Food Sources

Reports listing the *trans* fatty acid level in selected food items are available from the United States (Enig et al., 1990; Litin and Sacks, 1993; Michels and Sacks, 1995), Canada (Ratnayake et al., 1993), and Europe (Aro et al., 1998a, 1998b, 1998c; Michels and Sacks, 1995; van Erp-baart et al., 1998; van Poppel et al., 1998). More recently, a comprehensive U.S. database was compiled by the U.S. Department of Agriculture (ARS, 2001) that included a description of the methodology used to formulate the nutrient values (Schakel et al., 1997). *Trans* fatty acids are present in foods containing traditional stick margarine (3.04 g *trans* fatty acids/serving) and vegetable shortenings (2.54 g/serving) that have been subjected to hydrogenation, as well as in milk (0.22 g/serving), butter (0.40 g/serving) and meats (0.01 to 0.21 g/serving) (Emken, 1995). Therefore, foods that are contributors of *trans* fatty acids include pastries, fried foods (e.g., doughnuts and french fries), dairy products, and meats. Human milk contains approximately 1 to 5 percent of total energy as *trans* fatty acids (Table 8-7) and similarly, infant formulas contain approximately 1 to 3 percent (Ratnayake et al., 1997).

Dietary Intake

Estimating the amount of *trans* fatty acids in the food supply has been hampered by the lack of an accurate and comprehensive database from which to derive the data and the trend towards the reformulation of products over the past decade to reduce levels. This latter issue complicates analysis of historical food intake data. Additionally, the variability in the *trans* fatty acid content of foods within a food category is extensive and can introduce substantial error when the calculations are based on food frequency questionnaires that heavily rely on the grouping of similar foods (Innis et al., 1999). *trans* Fatty acid intake is not currently collected in U.S. national surveys.

Early reports suggested a wide range of *trans* fatty acid intakes, from 2.6 to 12.8 g/d (Emken, 1995). The lower estimated intakes tended to be derived from food frequency data, whereas the higher estimated intakes tended to be derived from food availability data. More recent data from food frequency questionnaires collected in the United States suggest average *trans* fatty acid intakes of 1.5 to 2.2 percent of energy (Ascherio et al., 1994; Hu et al., 1997), or 5.2 percent of total dietary fat (Lemaitre et al., 1998). Intakes of about 1 to 2 percent of energy have been reported for women in Canada, although the range of intakes was wide (Elias and Innis,

TABLE 8-7 *Trans* Fatty Acid Content in Term Human Milk of Women in the United States and Canada

Reference	Study Population/Stage of Lactation ^a	<i>Trans</i> Fatty Acid	Content in Human Milk	
			% of Total Fatty Acids	% of Total Energy ^b
Gibson and Kneebone, 1981	120 women, 40–45 d pp	16:1 18:1	trace ~ 10	trace ~ 5.46
Chappell et al., 1985	7 women, 1–37 d pp	18:1(9)	2.6 ± 0.4	1.42
		18:1(7)	0.1 ± 0.03	0.05
		18:1(5)	0.1 ± 0.04	0.05
		18:2(6) c,t+t,c ^c	0.1 ± 0.4	0.05
		Total	2.9	1.57
Chen et al., 1995a	198 samples, 3–4 wk pp	Total <i>trans</i>	7.19 ± 3.03	3.92
Innis and King, 1999	103 women, 2 mo pp	Total <i>trans</i>	7.1 ± 0.32	3.88

^a pp = postpartum.

^b Calculated using the following values: 40 g of fat/L of milk, 8.87 kcal/g of fat, 650 kcal/L of milk.

^c c,t+t,c = *cis*, *trans* and *trans*, *cis*.

2001, 2002). Most recently, *trans* fatty acid intake was estimated from existing CFSII data (Allison et al., 1999). The mean *trans* fatty acid intake for the U.S. population aged 3 years and older was 2.6 percent of total energy intake.

Conjugated Linoleic Acid

Food Sources

The average concentration of conjugated linoleic acid (CLA) in dairy products and ruminant meats is approximately 5 mg of CLA/g of fat (Chin et al., 1992). Although numerous CLA isomers have been reported to be found in meat, milk, and dairy products (Ha et al., 1989), the *cis*-9,*trans*-11 isomer is the predominant form of CLA present in these foods (Ma et al., 1999). The conjugated linoleic acid content of milk can vary depending on a number of factors, such as animal feed diet, pasture grazing, supple-

ment use, and number of lactations (MacDonald, 2000). Ma and coworkers (1999) reported values of 1.8 mg of CLA/g of fat for skim milk, 3.4 mg/g for whole milk, 4.3 mg/g for 1 percent milk, 5.0 mg/g for 2 percent milk, and 5.5 mg/g for half-and-half cream. In addition, values ranged from 2.7 to 6.2 mg of CLA/g of fat for various cheeses and 1.2 to 3.2 mg of CLA/g of fat for different types of raw and cooked beef products.

Dietary Intake

Recent analysis of duplicate food portions indicates CLA intake in the United States is in the range of 151 to 212 mg/d (Ritzenthaler et al., 2001). The average intake of *cis*-9,*trans*-11 octadecadienoic acid in a small group of Canadians was recently estimated to be about 95 mg/d (Ens et al., 2001). Based on the CLA content in the Health Canada National Nutritious Food Basket 1998 for purchased quantities, *cis*-9,*trans*-11 CLA intake for men and women was 332 and 295 mg/d, respectively. These values assume that all food purchased is actually eaten. From food records it is clear that the pattern of CLA intake is highly variable among individuals and from day-to-day for individuals themselves. Estimates from information on foods purchased, however, are higher than estimates from reported food intake data; therefore, the two data sets are not comparable.

ADVERSE EFFECTS OF OVERCONSUMPTION

Total Fat

A Tolerable Upper Intake Level (UL) was not set for total fat because of the lack of a defined intake level at which an adverse effect, such as obesity, can occur (see Chapter 11). An Acceptable Macronutrient Distribution Range (AMDR) for fat intake, however, has been estimated based on adverse effects from consuming low fat and high fat diets (Chapter 11).

Saturated Fatty Acids

Hazard Identification

Elevated LDL Cholesterol Concentration and Risk of CHD. Several hundred studies have been conducted to assess the effect of saturated fatty acids on serum cholesterol concentration. In general, the higher the intake of saturated fatty acids, the higher the serum total (Figure 8-2) and low density lipoprotein (LDL) cholesterol concentrations (Figure 8-3). Regression analyses of such studies have suggested that for each 1 percent increase

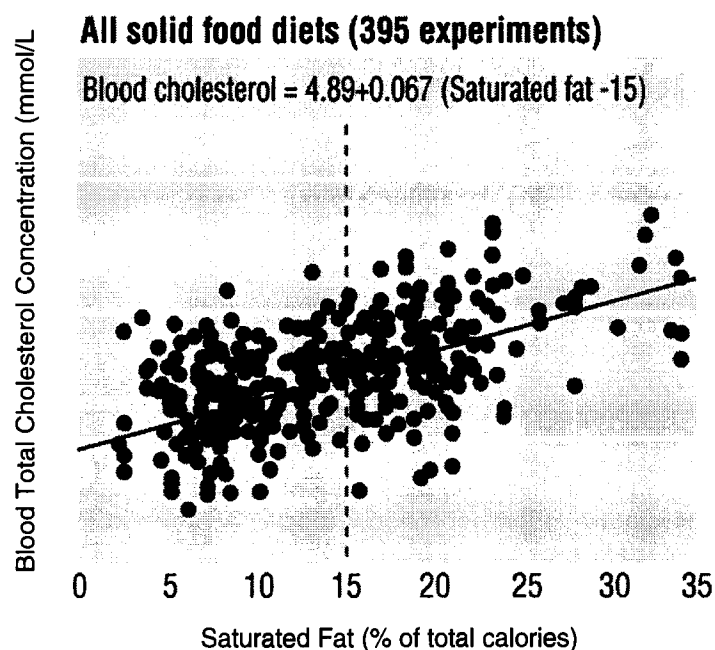


FIGURE 8-2 Relationship between blood total cholesterol concentrations and saturated fatty acid intake. Reprinted, with permission, from Clarke et al. (1997). Copyright 1997 by the *British Medical Journal*.

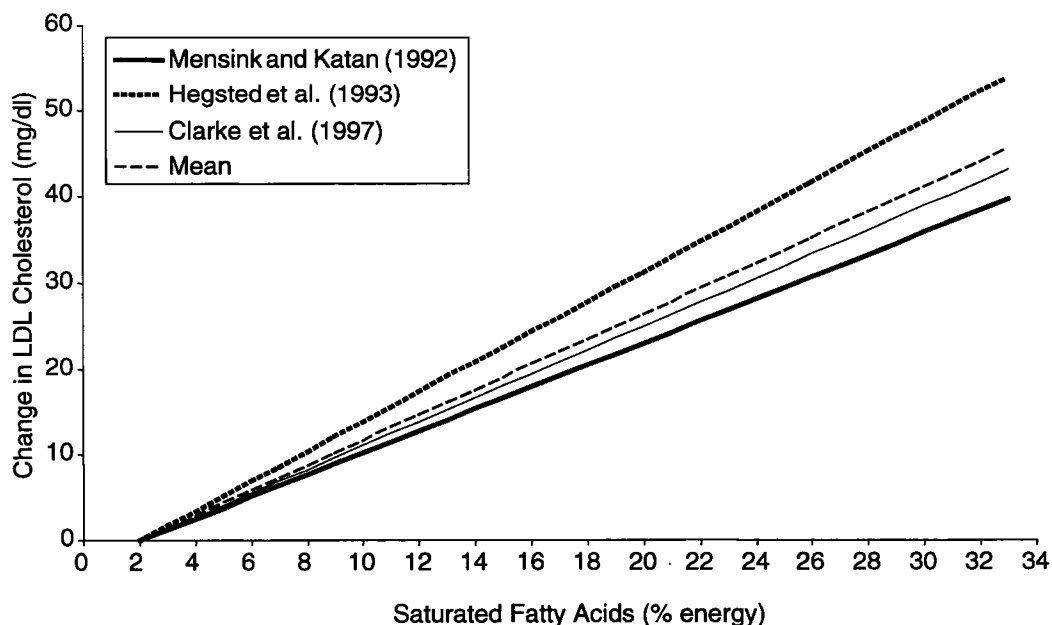


FIGURE 8-3 Calculated changes in serum low density lipoprotein cholesterol concentration in response to percent change in dietary saturated fatty acids. Three regression equations were used to establish the response curves. The range in saturated fatty acid intake was 2.2 to 33 percent of energy.

in energy from saturated fatty acids, serum LDL cholesterol concentration increases by 0.033 mmol/L (Mensink and Katan, 1992), 0.036 mmol/L (Clarke et al., 1997), or 0.045 mmol/L (Hegsted et al., 1993). Although all fats will increase serum high density lipoprotein (HDL) cholesterol concentration relative to carbohydrate, the increase attributable to saturated fats is greater than that observed for monounsaturated and polyunsaturated fatty acids. Serum HDL cholesterol concentration increases by 0.011 to 0.013 mmol/L for each 1 percent increase in saturated fat (Clarke et al., 1997; Hegsted et al., 1993; Mensink and Katan, 1992).

Similar to that observed for saturated fatty acid intake and LDL cholesterol concentration, there is a positive linear relationship between serum total and LDL cholesterol concentrations and risk of coronary heart disease (CHD) or mortality from CHD (Jousilahti et al., 1998; Neaton and Wentworth, 1992; Sorkin et al., 1992; Stamler et al., 1986; Weijenberg et al., 1996). Results from the Zutphen Elderly Study estimated that the relative risk of CHD mortality was 1.4 with a corresponding increase of 1 mmol/L of total serum cholesterol concentration (Weijenberg et al., 1996). It has been estimated that a 10 percent reduction in serum cholesterol concentration would reduce CHD mortality by 20 percent (Jousilahti et al., 1998).

A number of epidemiological studies have reported an association between saturated fatty acid intake and risk of CHD. The majority of these studies have reported a positive relationship between saturated fatty acid intake and risk of CHD and CHD mortality (Goldbourt et al., 1993; Hu et al., 1997, 1999a, 1999c; Keys et al., 1980; McGee et al., 1984). Ascherio and coworkers (1996) concluded that the association between saturated fatty acid intake and risk of CHD was not strong; however, saturated fat and the predicted effects on blood cholesterol concentrations did affect risk. No association between saturated fatty acid intake and coronary deaths was observed in the Zutphen Study or the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (Kromhout and de Lezenne Coulander, 1984; Pietinen et al., 1997).

Although all saturated fatty acids were originally considered to be associated with increased adverse health outcomes, including increased blood cholesterol concentrations, it later became apparent that saturated fatty acids differ in their metabolic effects (e.g., potency in raising blood cholesterol concentrations). In general, stearic acid has been shown to have a neutral effect on total and LDL cholesterol concentrations (Bonanome and Grundy, 1988; Denke, 1994; Hegsted et al., 1965; Keys et al., 1965; Yu et al., 1995; Zock and Katan, 1992). While palmitic, lauric, and myristic acids increase cholesterol concentrations (Mensink et al., 1994), stearic acid is more similar to oleic acid in its neutral effect (Kris-Etherton et al., 1993). Furthermore, a stearic acid-rich diet has been shown to improve

thrombogenic and atherogenic risk factor profiles (Kelly et al., 2001). However, it is impractical at the current time to make recommendations for saturated fatty acids on the basis of individual fatty acids.

Mortality. A number of studies have demonstrated a positive association between serum cholesterol concentration and the incidence of mortality (Conti et al., 1983; Corti et al., 1997; Haheim et al., 1993; Klag et al., 1993; Martin et al., 1986). Some studies, however, have reported an increased risk of non-CHD mortality, especially cancer, with low serum cholesterol concentration, suggesting a “U” or “J” shaped curve (Agner and Hansen, 1983; Frank et al., 1992; Kagan et al., 1981). The Poland and United States Collaborative Study on Cardiovascular Epidemiology showed an increased risk for cancer with low serum cholesterol concentrations in Poland, but not in the United States (Rywik et al., 1999). It was concluded that various nutritional and non-nutritional factors (obesity, smoking, alcohol use) were confounding factors, resulting in the differences observed between the two countries. As a specific example, body fat was shown to have a “U” shaped relation to mortality (Yao et al., 1991).

Obesity. A number of studies have attempted to ascertain the relationship between saturated fatty acid intake and body mass index, and these results are mixed. Saturated fatty acid intake was shown to be positively associated with body mass index or percent of body fat (Doucet et al., 1998; Gazzaniga and Burns, 1993; Larson et al., 1996; Ward et al., 1994). In contrast, no relationship was observed for saturated fatty acid intake and body weight (González et al., 2000; Ludwig et al., 1999; Miller et al., 1994).

Impaired Glucose Tolerance and Risk of Diabetes. Epidemiological studies have been conducted to ascertain the association between the intake of saturated fatty acids and the risk of diabetes. A number of these studies found no relationship (Colditz et al., 1992; Costa et al., 2000; Salmerón et al., 2001; Sevak et al., 1994; Virtanen et al., 2000). Several large epidemiological studies, however, showed increased risk of diabetes with increased intake of saturated fatty acids (Feskens et al., 1995; Hu et al., 2001; Marshall et al., 1997; Parker et al., 1993). The Normative Aging Study found that a diet high in saturated fatty acids was an independent predictor for both fasting and postprandial insulin concentration (Parker et al., 1993). A reduction in saturated fatty acid intake from 13.9 to 7.8 percent of energy was associated with an 18 percent decrease in fasting insulin and a 25 percent decrease in postprandial insulin concentrations.

Findings from short-term intervention studies tend to suggest a lack of adverse effect of saturated fatty acids on risk indicators for diabetes in

healthy individuals. Postprandial glucose and insulin concentrations were not significantly different in men who ingested three different levels of saturated fatty acids (Roche et al., 1998). Fasching and coworkers (1996) reported no difference in insulin secretion or sensitivity in men who consumed a 33 percent saturated, monounsaturated, or polyunsaturated fatty acid diet. There was no difference in postprandial glucose or insulin concentration when healthy adults were fed butter or olive oil (Thomsen et al., 1999). Louheranta and colleagues (1998) found no difference in glucose tolerance and insulin sensitivity in healthy women fed either a high oleic or stearic acid diet. In contrast, results of the KANWU study indicate that consumption of high levels (18 percent of energy) of saturated fats can significantly impair insulin sensitivity (Vessby et al., 2001).

Summary

Intakes above an identified UL indicate a potential risk of an adverse health effects. There is a positive linear trend between total saturated fatty acid intake and total and LDL cholesterol concentration and increased risk of CHD. A UL is not set for saturated fatty acids because any incremental increase in saturated fatty acid intake increases CHD risk. It is neither possible nor advisable to achieve 0 percent of energy from saturated fatty acids in typical whole-food diets. This is because all fat and oil sources are mixtures of fatty acids, and consuming 0 percent of energy would require extraordinary changes in patterns of dietary intake, such as the inclusion of fats and oils devoid of saturated fatty acids, which are presently unavailable. Such extraordinary adjustments may introduce undesirable effects (e.g., inadequate intakes of protein and certain micronutrients) and unknown and unquantifiable health risks. It is possible to consume a diet low in saturated fatty acids by following the dietary guidance provided in Chapter 11.

Cis-Monounsaturated Fatty Acids

Hazard Identification

Cardiovascular Disease. Within the range of usual intake, there are no clearly established adverse effects of *n*-9 monounsaturated fatty acids in humans. There is some preliminary evidence that a meal providing 50 g of fat from olive oil reduced brachial artery flow-mediated vasodilation by 31 percent in 10 healthy, normolipidemic individuals versus canola oil or salmon (Vogel et al., 2000). In addition, there is evidence from nonhuman primates that a diet rich in *n*-9 monounsaturated fatty acids promotes

atherosclerosis just as much as a diet containing isocaloric amounts of saturated or polyunsaturated fatty acids (Rudel et al., 1997). Dietary monounsaturated fatty acids induce atherogenesis due to greater hepatic lipid concentrations (i.e., triacylglycerol, free cholesterol, and cholesteryl ester), as well as the high degree of cholesteryl oleate enrichment in plasma cholesteryl esters. Overconsumption of energy related to a high *n*-9 monounsaturated fatty acid and high fat diet is another potential risk associated with excess consumption of monounsaturated fatty acids. *n*-9 Monounsaturated fatty acid intake may result in an increase in energy intake from saturated fatty acids due to the simultaneous occurrence of saturated and *n*-9 monounsaturated fatty acids in animal fats.

The *n*-7 monounsaturated fatty acid, palmitoleic acid, behaves like saturated fatty acids in raising LDL cholesterol concentration (Nestel et al., 1994). Watts and coworkers (1996) reported a positive correlation between palmitoleic acid and progression of CHD.

Cancer. While most epidemiological studies indicate that monounsaturated fatty acid intake is not associated with increased risk of most cancers (Holmes et al., 1999; Hursting et al., 1990; van Dam et al., 2000; van den Brandt et al., 1993), a few studies have observed a positive association. There is some epidemiological evidence for a positive association between oleic acid intake and breast cancer risk in women with no history of benign breast disease (Velie et al., 2000). In addition, one study reported that women with a family history of colorectal cancer who consumed a diet high in mono- and polyunsaturated fatty acids were at greater risk of colon cancer than women without a family history (Slattery et al., 1997). Giovannucci and coworkers (1993) reported a positive association between monounsaturated fatty acid intake and risk of advanced prostate cancer, while two studies observed increased risk of lung cancer (De Stefani et al., 1997; Veierød et al., 1997).

Summary

Based on the lack of adequate data on adverse effects of monounsaturated fatty acids, a UL is not set.

n-6 Polyunsaturated Fatty Acids

A UL is not set for *n*-6 polyunsaturated fatty acids because of the lack of a defined intake level at which an adverse effect can occur (see Chapter 11). An AMDR for *n*-6 polyunsaturated fatty acids, however, is estimated based on adverse effects from consuming a diet low or high in *n*-6 polyunsaturated fatty acids (Chapter 11).

n-3 Polyunsaturated Fatty Acids

Because the longer-chain *n*-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are biologically more potent than their precursor, α -linolenic acid, much of the work on the adverse effects of this group of fatty acids has been on DHA and EPA.

Hazard Identification

Immune Function. Numerous studies have shown suppression of various aspects of human immune function in vitro or ex vivo in peripheral blood mononuclear cells, or in isolated neutrophils or monocytes in individuals provided *n*-3 polyunsaturated fatty acids as a supplement or as an experimental diet compared with baseline values before the intervention (Table 8-8). The minimum dose observed for such an effect was 0.9 g/d of EPA and 0.6 g/d of DHA given as fish oil for 6 to 8 weeks to healthy adults (Cooper et al., 1993). The level of EPA that caused some type of immunosuppression ranged from 0.9 to 9.4 g/d when fed for 3 to 24 weeks. The level of DHA that caused immunosuppression ranged from 0.6 to 6.0 g/d (Table 8-8).

The data in single treatment studies comparing baseline versus post-supplementation immune function indicate that *n*-3 polyunsaturated fatty acids, especially EPA and DHA at levels 7 to 15 times greater than typical current U.S. intakes, diminish the potential of the immune system to attack pathogens (Kelley et al., 1998, 1999; Lee et al., 1985; Schmidt et al., 1989). This diminished ability, however, is also associated with suppression of inflammatory responses, suggesting benefits for individuals suffering from autoimmune diseases such as rheumatoid arthritis. It seems that the same doses of *n*-3 fatty acids that may be beneficial in chronic disease prevention are doses that are also immunosuppressive.

Several studies using a design of comparison across treatment groups (Blok et al., 1997; Kelley et al., 1998; Mølviq et al., 1991; Yaqoob et al., 2000), rather than comparison within individuals with a baseline, have shown a lack of several potential adverse effects of EPA and DHA supplementation on human immune cell functions. In one key study, 58 healthy men were given daily supplements of 0, 3, 6, or 9 g/d of a fish-oil supplement (EPA intake of 0, 0.81, 1.62, or 2.43 g/d and DHA intake of 0, 0.16, 0.33, or 0.49 g/d) for 1 year (Blok et al., 1997). Ex vivo endotoxin-stimulated production of interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , or IL-1Ra (IL-1 receptor antagonist) did not differ among treatments up to 6 months after the fish-oil supplementation was stopped. These data support a lack of long-term adverse effect of fish-oil supplementation on cytokine activity.

TABLE 8-8 Effects of *n*-3 Fatty Acid Intake on Immune Function

Reference	Study Design	<i>n</i> -3 Fatty Acid Dose (Daily) ^a
Lee et al., 1985	7 men 6 wk	MaxEPA (3.2 g EPA, 2.2 g DHA)
Endres et al., 1989	9 men 6 wk	MaxEPA (2.75 g EPA, 1.85 g DHA)
Schmidt et al., 1989	12 men 6 wk	Cod liver oil (2.5 g EPA)
Kelley et al., 1991	10 men 56-d crossover	Basal diet Flaxseed oil-supplemented diet (20 g 18:3 <i>n</i> -3)
Meydani et al., 1991	6 young women, 6 older women 12 wk	ProMega (1.68 g EPA, 0.72 g DHA)
Mølviq et al., 1991	8 men 9 men 8 men 7 wk	Placebo oil Fish oil (1 g EPA, 0.5 g DHA) Fish oil (2 g EPA, 1 g DHA)
Thompson et al., 1991	6 men, 6 women 4-wk crossover	MaxEPA (2.16 g EPA) 12 g olive oil
Virella et al., 1991	4 men fed fish oil, 2 men fed olive oil 6 wk	Fish oil (2.4 g EPA)
Yamashita et al., 1991	3 adults 1 d	3 g EPA, infused
Cooper et al., 1993	8 men and women 6–8 wk	Fish oil (0.9 g EPA, 0.6 g DHA)
Endres et al., 1993	9 men 6 wk	MaxEPA (2.75 g EPA, 1.85 g DHA)
Meydani et al., 1993	7 women, 3 men 24 wk after 6 wk on typical U.S. diet (baseline)	Low fat, high fish diet (1.23 g EPA + DHA)
Sperling et al., 1993	5 women and 3 men with rheumatoid arthritis 10 wk	SuperEPA (9.4 g EPA, 5.0 g DHA)

 Results^b

- Depressed neutrophil LTB₄, 6-*trans*-LTB₄, 5-HETE, and endothelial adherence, monocyte LTB₄ and 5-HETE, neutrophil chemotaxis
- Depressed PBMC IL-1 β , IL-1 α , TNF, PGE₂, and neutrophil chemotaxis
- Depressed neutrophil migration, monocyte cell density (marker of monocyte migration)
- Depressed PBMC proliferation in response to T-cell mitogen but not to B-cell mitogen with flaxseed oil-supplemented diet
- Depressed PBMC IL-1 β and IL-6 (greater in older women), TNF and IL-2 (older women only)
- Depressed PBMC proliferation, IL-1 β in PBMCs and monocytes with *n*-3 fatty acids
PBMC secretion of IL-1 β , TNF- α , PGE₂ or LTB₄ not affected by *n*-3 fatty acids
- Depressed neutrophil chemiluminescence (marker of neutrophil function) with MaxEPA diet
- Depressed PBMC IL-2
- Depressed NK cell activity of PBMCs
- Typhoid vaccine injection site less inflamed, postvaccination tachycardia inhibited, depressed blood IL-1 and IL-6 concentrations
- Depressed PBMC IL-2 and proliferation
- Depressed PBMC IL-1 β , TNF, IL-6, PGE₂, CD₄⁺ lymphocytes, and lymphocyte proliferation, delayed-type hypersensitivity
- Depressed neutrophil chemotaxis, inositol tris-phosphate formation, and LTB₄, monocyte LTB₄

continued

TABLE 8-8 Continued

Reference	Study Design	<i>n</i> -3 Fatty Acid Dose (Daily) ^a
Gallai et al., 1995	20 patients with relapsing/remitting multiple sclerosis and 15 controls 6 mo	Fish oil (3.06 g EPA, 1.86 g DHA)
Caughey et al., 1996	30 men 4-wk diet + 4-wk diet with fish oil	Flaxseed oil-enriched diet and fish oil (EPA 1.62 g, DHA 1.08 g) Sunflower oil diet and fish oil (EPA 1.62 g, DHA 1.08 g)
Hughes et al., 1996	3 men, 3 women 3 wk	EPA Forte (0.93 g EPA, 0.63 g DHA)
Blok et al., 1997	58 men 1 y	0, 3, 6, or 9 g fish oil (0, 0.81, 1.62, or 2.43 g EPA; 0, 0.16, 0.33, or 0.49 g DHA)
Kelley et al., 1998	4 men 7 men 120 d	Basal diet DHA-enriched oil (6 g DHA)
Kelley et al., 1999	4 men 7 men 120 d	Basal diet DHA-enriched oil (6 g DHA)
Yaqoob et al., 2000	5 men, 3 women 7 men, 1 woman 3 other groups of 8 fed other oils, but all comparable to placebo 12-wk parallel	Placebo oil (3:1 coconut and soybean oils) Fish oil (2.1 g EPA, 1.1 g DHA)

^a EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid.

^b LTB₄ = leukotriene B₄, 5-HETE = 5-hydroxyeicosatetraenoic acid, PBMC = peripheral blood mononuclear cell, IL = interleukin, TNF = tumor necrosis factor, PGE₂ = prosta-

In studies using multitreatment parallel designs, potential adverse effects of *n*-3 fatty acids on immune function that were observed include decreased expression of monocyte major histocompatibility complex antigens and cell surface adhesion proteins (Hughes et al., 1996), decreased peripheral blood mononuclear cell (PBMC) proliferation and IL-1 β in

Results^b

Depressed PBMC IL-1 β , TNF- α , IL-2 and IFN- γ , PGE₂, and LTB₄,
serum-soluble IL-2 receptors

Depressed PBMC TNF- α , IL-1 β , TxB₂, and PGE₂ with flaxseed
oil-enriched diet

Greater decreases in PBMC TNF- α , IL-1 β , and TxB₂ in both groups
after fish-oil supplementation

Depressed monocyte surface proteins: HLA-DR, HLA-DP, HLA-DQ,
ICAM-1, LFA-1

No effect on whole blood IL-1 β , TNF- α , or IL-1 receptor antagonist

Decreased white blood cells

PBMC proliferation and delayed-type hypersensitivity not different
between groups

Depressed PBMC IL-1 β and TNF- α production, in vitro PBMC PGE₂
and LTB₄ secretion

No effect of fish oil on PBMC NK cell activity, proliferation, types of
blood lymphocytes, IL-1 α , IL-1 β , TNF- α , IL-2, IL-10, and IFN- γ

glandin E₂, NK cell = natural killer cell, IFN- γ = interferon- γ , TxB₂ = thromboxane B₂,
HLA = human leukocytes antigen, ICAM = intercellular adhesion molecule, LFA =
leukocyte function-associated antigen.

PBMCs and monocytes (Mølviq et al., 1991), decreased PBMC IL-2 (Virella
et al., 1991), decreased but still clinically normal neutrophils (Kelley et al.,
1998), and decreased tachycardia and inflammation after typhoid vaccine
(Cooper et al., 1993).

All of the single treatment studies comparing individuals fed *n*-3 polyunsaturated fatty acids before and after supplementation showed immunosuppressive effects. Differences in study design (single treatment versus multitreatment parallel designs) seem to be quite significant in determining whether *n*-3 fatty acid supplementation exerts immunosuppression or not. There is no clear basis to prefer one type of study design to the other. For example, the difference in results between Caughey and colleagues (1996) (a baseline comparison study) and Blok and colleagues (1997) (a group comparison study) is not accounted for by greater variability in measurements by the latter group. The standard deviation for whole blood TNF- α was no more than 5 percent of the mean in the study by Blok and coworkers (1997), and the standard deviation for mononuclear cell TNF- α was 25 to 45 percent of the mean in the study by Caughey and coworkers (1996). In another study using intertreatment comparisons of control versus men given fish oil for 7 weeks, secretions of IL-1 β and TNF- α were not suppressed by fish-oil feeding, but lysates of peripheral blood mononuclear cells from people given fish oil contained less IL-1 β and TNF- α than did cells from controls (Mølviq et al., 1991). Therefore, the study by Mølviq and colleagues (1991) showed some concurrence with that of Blok and colleagues (1997) and Caughey and colleagues (1996).

Another alternative is to extrapolate from animal studies using model species that are known to have similar immune system components and responsiveness compared to humans. Detailed characterization of appropriateness of animal models for extrapolation to humans with respect to immunosuppression has not been done. A few animal studies have shown the effects of dietary *n*-3 fatty acids on response to infection (Chang et al., 1992; Fritsche et al., 1997). At this time, there are not sufficient data to support establishing an UL for EPA and DHA based on infection responsiveness.

Bleeding and Increased Risk of Hemorrhagic Stroke. One of a number of factors that has been suggested to link *n*-3 polyunsaturated fatty acid intake with reduced risk of CHD is reduced platelet aggregation, and therefore prolonged bleeding time. The platelet count can decline by as much as 35 percent; however, the count does not usually fall below the lower limit of normal (Goodnight et al., 1981). Although prolonged bleeding times have been shown to be beneficial in preventing heart disease, bleeding times can become prolonged enough to result in excessive bleeding and bruising. Intervention studies that have examined the effects of *n*-3 fatty acids on bleeding time are mixed. A number of short-term studies (4 to 11 weeks) have shown significant increased bleeding time with taking EPA/DHA supplements ranging from 2 to 15 g/d (Cobiac et al., 1991; De

Caterina et al., 1990; Levinson et al., 1990; Lorenz et al., 1983; Mortensen et al., 1983; Sanders et al., 1981; Schmidt et al., 1990, 1992; Smith et al., 1989; Thorngren and Gustafson, 1981; Wojenski et al., 1991; Zucker et al., 1988), whereas other studies using similar intake levels resulted in no difference (Blonk et al., 1990; Freese and Mutanen, 1997; Rogers et al., 1987). Analysis of these studies collectively indicated no dose-response for EPA and DHA intake and the percent increase in bleeding time. Schmidt and coworkers (1992) reported increased bleeding times when 3.1 g/d of EPA and DHA were given for 6 weeks and 9 months. None of the above studies reported excessive bleeding times, bleeding episodes, or bruising.

Dietary feeding studies that provided approximately 2 percent of energy as EPA and DHA from salmon did not result in increased bleeding time compared to a stabilization diet that contained only 0.3 percent of energy as EPA and DHA (Nelson et al., 1991). Excessive cutaneous bleeding time and reduced in vitro platelet aggregability have been reported in Greenland Eskimos (Dyerberg and Bang, 1979; Dyerberg et al., 1978) who ingest on average 6.5 g/d (3.8 percent of energy) of EPA and DHA derived mainly from seal (Bang et al., 1980). A tendency to bleed from the nose and urinary tract was observed among the Greenland Eskimos (Bang and Dyerberg, 1980). One study comparing perirenal adipose tissue fatty acid profiles with incidence of hemorrhagic stroke in human autopsy cases from Greenland showed that the amounts of EPA and DHA in the adipose tissue of 4 hemorrhagic stroke victims was greater than in 26 control cases with no cerebral pathology (Pedersen et al., 1999). Furthermore, ecological studies have suggested an increased risk of hemorrhagic stroke among Greenland Eskimos (Kristensen, 1983; Kromann and Green, 1980). A recent prospective study in the United States showed no association between intake of *n*-3 fatty acids and risk of hemorrhagic stroke (Iso et al., 2001). The median intake levels for the quintiles of *n*-3 polyunsaturated fat intake, however, ranged from only 0.077 to 0.481 g/d, which reflects the relatively low intake level of *n*-3 fatty acids in the United States.

Oxidative Damage. Long-chain polyunsaturated fatty acids, particularly DHA and EPA, are vulnerable to lipid peroxidation, resulting in oxidative damage of various tissues. Numerous feeding studies using laboratory animals have demonstrated increased lipid peroxidation and oxidative damage of erythrocytes, liver, and kidney membranes and bone marrow DNA with consumption of DHA (Ando et al., 1998; Song and Miyazawa, 2001; Umegaki et al., 2001; Yasuda et al., 1999). The oxidative damage was shown to be reduced or prevented with the coconsumption of vitamin E (Ando et al., 1998; Leibovitz et al., 1990; Yasuda et al., 1999).

Summary

While there is evidence to suggest that high intakes of *n*-3 polyunsaturated fatty acids, particularly EPA and DHA, may impair immune response and result in excessively prolonged bleeding times, it is not possible to establish a UL. Studies on immune function were done in vitro and it is difficult, if not impossible, to know how well these artificial conditions simulate human immune cell response in vivo. Data on EPA and DHA intakes and bleeding times are mixed and a dose-response effect was not observed. Although excessively prolonged bleeding times and increased incidence of bleeding have been observed in Eskimos, whose diets are rich in EPA and DHA, information is lacking to conclude that EPA and DHA were the sole basis for these observations. At the 99th percentile of intake, the highest intakes of dietary EPA and DHA were 0.662 and 0.651 g/d, respectively, in men 71 years of age and older (Appendix Tables E-12 and E-14). This EPA + DHA intake (1.31 g/d) is much lower than that for Greenland Eskimos (6.5 g/d). EPA and DHA are available as dietary supplements, and until more information is available on the adverse effects of EPA and DHA, these supplements should be taken with caution.

Special Considerations

A few special populations have been reported to exhibit adverse effects from consuming *n*-3 polyunsaturated fatty acids. Despite the favorable effects of *n*-3 fatty acids on glucose homeostasis, caution has been suggested for the use of *n*-3 fatty acids in those individuals who already exhibit glucose intolerance or diabetic conditions (Glauber et al., 1988; Kasim et al., 1988) that require increased doses of hypoglycemic agents (Friday et al., 1989; Stacpoole et al., 1989; Zambon et al., 1992). Increased episodes of nose bleeds have been observed in individuals with familial hypercholesterolemia during fish-oil supplementation (Clarke et al., 1990). Anticoagulants, such as aspirin, warfarin, and coumadin, will prolong bleeding times and the simultaneous ingestion of *n*-3 fatty acids by individuals may excessively prolong bleeding times (Thorngren and Gustafson, 1981). Therefore, the subpopulations described above should take supplements containing EPA and DHA with caution.

Trans Fatty Acids

Hazard Identification

Total and LDL Cholesterol Concentrations. Prior to 1980 there was generally little concern about the trend toward increased consumption of

hydrogenated fat in the U.S. diet, especially when the hydrogenated fats displaced fats relatively high in saturated fatty acids (Denke, 1995). During the early 1980s studies showed a hypercholesterolemic effect of *trans* fatty acids in rabbits (Kritchevsky, 1982; Ruttenberg et al., 1983). Renewed interest in the topic of hydrogenated fat in human diets, or more precisely *trans* fatty acid intake, started in the early 1990s. The availability of a methodology to distinguish the responses of individual lipoprotein classes to dietary modification expanded the depth to which the topic could be readdressed.

A report from the Netherlands suggested that a diet enriched with elaidic acid (a subfraction of 18:1 *trans*) compared to one enriched with oleic acid (18:1 *cis*) increased total and LDL cholesterol concentrations and decreased HDL cholesterol concentrations, hence resulting in a less favorable total cholesterol:HDL cholesterol ratio (Mensink and Katan, 1990). Consumption of a diet enriched with saturated fatty acids resulted in LDL cholesterol concentrations similar to those observed after individuals consumed the diet high in elaidic acid, but HDL cholesterol concentrations were similar to those observed after individuals consumed the diet high in oleic acid. A number of similar studies have been published since then and have reported that hydrogenated fat/*trans* fatty acid consumption increases LDL cholesterol concentrations (Aro et al., 1997; Judd et al., 1994, 1998; Louheranta et al., 1999; Müller et al., 1998; Sundram et al., 1997) (Tables 8-9, 8-10, and 8-11). Recent data have demonstrated a dose-dependent relationship between *trans* fatty acid intake and the LDL:HDL ratio and when combining a number of studies, the magnitude of this effect is greater for *trans* fatty acids compared with saturated fatty acids (Figure 8-4) (Ascherio et al., 1999).

Similar to the metabolic clinical trial data, studies in free-living individuals asked to substitute hydrogenated fat for other fat in their habitual diet resulted in higher concentrations of total and LDL cholesterol (Table 8-11) (Nestel et al., 1992b; Noakes and Clifton, 1998; Seppänen-Laakso et al., 1993).

No studies have been conducted to evaluate the effect of *trans* fatty acids that are present in meats and dairy products on LDL concentrations. The relative effect of *trans* fatty acids in meat and dairy products on LDL cholesterol concentration would be small compared to hydrogenated oils because of the lower levels that are present, and because any rise in concentration would most likely be due to the abundance of saturated fatty acids.

HDL Cholesterol Concentrations. The data related to the impact of hydrogenated fat/*trans* fatty acids compared with unhydrogenated oil/*cis* fatty acids on HDL cholesterol concentrations are less consistent than for LDL cholesterol concentrations (Tables 8-9, 8-10, and 8-11). As reported

TABLE 8-9 Dietary *Trans* Fatty Acids (TFA) and Blood Lipid Concentration: Controlled Feeding Trials

Reference	Study Population	Diet ^a
Mensink and Katan, 1990; Mensink et al., 1992	79 men and women, avg 25–26 y	3-wk crossover, 40% fat 10% 18:1 10% SF 10% TFA
Zock and Katan, 1992	56 healthy men and women	3 wk crossover, 41% fat 18:2 18:0 TFA
Judd et al., 1994	58 men and women	6-wk crossover, 40% fat 18:1 SFA moderate TFA high TFA
Aro et al., 1997	80 healthy men and women, 20–52 y	5-wk intervention, 33% fat 18:0 TFA
Sundram et al., 1997	27 men and women, 19–39 y	4-wk crossover, 31% fat 18:1 16:0 12:0 + 14:0 TFA
Louheranta et al., 1999	14 healthy women, avg 23 y	4-wk crossover, 37% fat 18:1 TFA
Judd et al., 2002	50 men	5-wk crossover, 39% fat 18:1 18:0 TFA/18:0 TFA

^a SF = saturated fat, SFA = saturated fatty acids.

^b LDL-C = low density lipoprotein cholesterol, HDL-C = high density lipoprotein cholesterol, Lp(a) = lipoprotein(a).

TFA (% of energy)	Blood Lipid Concentrations ^b		
	LDL-C (mmol/L)	HDL-C (mmol/L)	Lp(a) (mg/L)
0	2.67 ^c	1.42 ^c	32 ^c
1.8	3.14 ^d	1.42 ^c	26 ^d
10.9	3.04 ^e	1.25 ^d	45 ^e
0.1	2.83 ^c	1.47 ^c	
0.3	3.00 ^d	1.41 ^d	
7.7	3.07 ^d	1.37 ^d	
0.7	3.34 ^c	1.42 ^c	
0.7	3.64 ^d	1.40 ^{c,d}	
3.8	3.54 ^e	1.47 ^e	
6.6	3.60 ^{d,e}	1.38 ^d	
0.4	2.89 ^c	1.42 ^c	270 ^c
8.7	3.13 ^d	1.22 ^d	308 ^d
0	3.17	1.25	128.3
0	3.15	1.26	122.0
0	3.57	1.18	134.3
6.9	3.81	1.05	153.3
0	2.53	1.37	225 (units/L)
5.1	2.64	1.31	220 (units/L)
0	2.95 ^c		
0	3.10 ^d		
4	3.32 ^e		
8	3.36 ^e		

^{c,d,e} Within each study, LDL-C, HDL-C, or Lp(a) concentrations that are significantly different between treatment groups have a different superscript.

TABLE 8-10 Hydrogenated Fat Intake and Blood Lipid Concentrations: Controlled Feeding Trials

Reference	Study Population	Diet ^a
Lichtenstein et al., 1993	14 men and women, 44–78 y	32-d crossover, 30% fat Baseline Corn oil Corn oil margarine
Almendingen et al., 1995	31 men, 21–46 y	3-wk crossover, 33–36% fat Butter PHFO PHSO
Judd et al., 1998b	46 men and women, 28–65 y	5-wk crossover, 34% fat PUFA-M Butter TFA-M
Müller et al., 1998	16 healthy females, 19–30 y	14-d crossover, 31–32% fat Vegetable oil PHFO
Lichtenstein et al., 1999	36 men and women, > 50 y	35-d crossover, 30% fat Soybean oil Semiliquid margarine Butter Soft margarine Shortening Stick margarine

^a PHFO = partially hydrogenated fish oil, PHSO = partially hydrogenated soybean oil, PUFA-M = margarine containing polyunsaturated fatty acids, TFA-M = margarine containing *trans* fatty acids.

^b TFA = *trans* fatty acids.

for LDL cholesterol concentrations, the effect of hydrogenated fat/*trans* fatty acids on HDL cholesterol concentrations, if present, is likely to be dose-dependent (Judd et al., 1994). The preponderance of the data suggests that hydrogenated fat/*trans* fatty acids, relative to saturated fatty acids, result in lower HDL cholesterol concentrations (Ascherio et al., 1999; Zock and Mensink, 1996; Zock et al., 1995). Because of the potentially

TFA ^b (% of energy)	Blood Lipid Concentrations ^c		
	LDL-C (mmol/L)	HDL-C (mmol/L)	Lp(a) (mg/L)
0.77	3.96 ^d	1.24 ^d	140 ^d
0.44	3.23 ^e	1.14 ^e	160 ^d
4.16	3.49 ^e	1.11 ^e	130 ^d
0.9	3.81 ^d	1.05 ^d	194 ^d
8.0	3.94 ^{d,f}	0.98 ^e	234 ^e
8.5	3.58 ^e	1.05 ^d	238 ^e
2.4	3.21 ^d	1.24 ^d	197 ^d
2.7	3.44 ^e	1.27 ^d	186 ^e
3.9	3.27 ^f	1.24 ^d	202 ^d
1.1	2.63 ^d	1.32 ^d	212 ^d
1.7	2.87 ^e	1.28 ^d	225 ^d
0.55	3.98 ^d	1.11 ^{d,e}	230
0.91	4.01 ^{d,e}	1.11 ^{d,e}	230
1.25	4.58 ^f	1.16 ^e	220
3.30	4.11 ^{d,e}	1.11 ^{d,e}	240
4.15	4.24 ^e	1.11 ^{d,e}	240
6.72	4.34 ^e	1.01 ^d	240

^c LDL-C = low density lipoprotein cholesterol, HDL-C = high density lipoprotein cholesterol, Lp(a) = lipoprotein(a).

^{d,e,f} Within each study, LDL-C, HDL-C, or Lp(a) concentrations that are significantly different between treatment groups have a different superscript.

differential effects of hydrogenated fat/*trans* fatty acids on LDL and HDL cholesterol concentrations, concern has been raised regarding their effect on the total cholesterol or LDL cholesterol:HDL cholesterol ratio (Ascherio et al., 1999). However, with respect to dietary fat recommendations, the strategy to improve the total cholesterol or LDL cholesterol:HDL

TABLE 8-11 Dietary *Trans* Fatty Acids (TFA), Hydrogenated Fat, and Blood Lipid Concentrations: Free-Living Trials

Reference	Study Population	Diet ^a
Nestel et al., 1992a	26 mildly hypercholesterolemic men, 27–57 y	4-wk crossover, 42% fat Control 1 Control 2 Blend 1 Blend 2
Nestel et al., 1992b	27 mildly hypercholesterolemic men, 30–63 y	3-wk crossover, 36–37% fat Control 18:1 TFA 16:0
Seppänen- Laakso et al., 1993	57 men and women, middle-aged	12-wk crossover to 1 of 2 diets, 39–43% fat Margarine Rapeseed Olive oil
Wood et al., 1993a	38 healthy men, 30–60 y	6-wk crossover, 38% fat Butter Butter-sunflower Butter-olive Hard margarine Soft margarine
Wood et al., 1993b	29 healthy men, 30–60 y	6-wk crossover, 37% fat Butter Crude palm Margarine Refined palm Refined palm+sunflower Sunflower oil
Chisholm et al., 1996	49 hypercholesterolemic men and women, avg 47 y	6-wk crossover, 26–27% fat Butter Margarine

TFA (% of energy)	Blood Lipid Concentrations ^c		
	LDL-C (mmol/L)	HDL-C (mmol/L)	Lp(a) (units/L)
3.8	4.13 ^c	1.11 ^c	
3.7	4.03 ^{c,d}	1.15 ^c	
6.7	3.92 ^{d,e}	1.10 ^c	
6.6	3.83 ^e	1.11 ^c	
< 1	4.22 ^c	0.98 ^c	235 ^c
1.4	3.90 ^d	0.98 ^c	236 ^c
5.7	4.27 ^c	0.98 ^c	296 ^d
< 1	4.16 ^c	1.09 ^d	249 ^e
	Change from baseline	Change from baseline	
2.9	-0.20	+0.05	
0	-0.30	-0.01	
0	-0.32	0.00	
2.1	3.78 ^c	1.22 ^c	
1.0	3.49 ^d	1.19 ^c	
1.0	3.59 ^d	1.22 ^c	
11.1	3.47 ^d	1.16 ^c	
0	3.26 ^e	1.16 ^c	
0.2	3.52 ^c	1.03 ^c	
0	3.36 ^c	1.03 ^c	
3.0	3.36 ^c	1.00 ^c	
0	3.41 ^c	1.06 ^d	
0	3.41 ^c	1.03 ^c	
0	3.23 ^d	1.00 ^c	
1.4	4.21 ^c	1.26 ^c	223 ^c
3.6	3.82 ^d	1.24 ^c	249 ^c

continued

TABLE 8-11 Continued

Reference	Study Population	Diet ^a
Noakes and Clifton, 1998	38 mildly hyperlipidemic men and women	3-wk crossover, 2 groups, 31–35% fat Canola + TFA TFA-free canola Butter PUFA + TFA TFA-free PUFA Butter

^a PUFA = polyunsaturated fatty acids.

^b LDL-C = low density lipoprotein cholesterol, HDL-C = high density lipoprotein cholesterol, Lp(a) = lipoprotein(a).

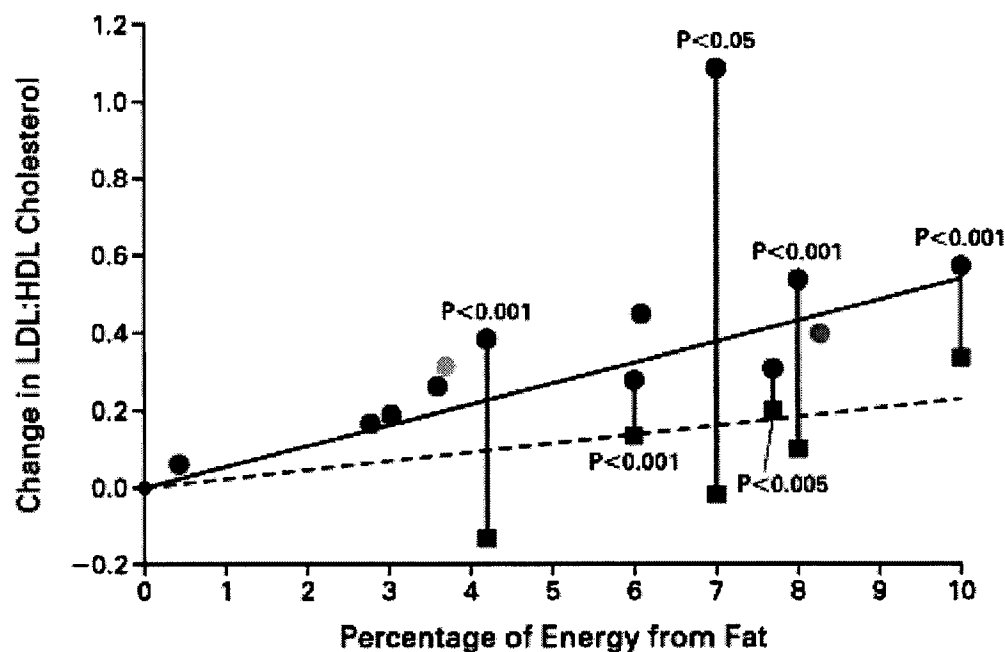


FIGURE 8-4 Change in the low density lipoprotein (LDL):high density lipoprotein (HDL) cholesterol concentration with increasing energy intake from saturated and *trans* fatty acids. Solid line represents the best-fit regression for *trans* fatty acids. Dotted line represents the best-fit regression for saturated fatty acids. Reprinted, with permission, from Ascherio et al. (1999). Copyright 1999 by the Massachusetts Medical Society.

TFA (% of energy)	Blood Lipid Concentrations ^c		
	LDL-C (mmol/L)	HDL-C (mmol/L)	Lp(a) (units/L)
3.3	3.64 ^c	1.19 ^c	
0	3.61 ^c	1.28 ^c	
1.1	4.14 ^d	1.20 ^c	
3.6	4.23 ^c	1.17 ^c	
0	3.98 ^d	1.23 ^c	
1.2	4.70 ^e	1.27 ^c	

^{c,d,e} Within each study, LDL-C, HDL-C, or Lp(a) concentrations that are significantly different between treatment groups have a different superscript.

cholesterol ratio would not be different from that to decrease LDL cholesterol concentrations.

Lp(a) Concentrations. Lipoprotein(a) (Lp(a)) concentrations in plasma have been associated with increased risk for developing cardiovascular and cerebrovascular disease, possibly via inhibition of plasminogen activity (Lippi and Guidi, 1999; Nielsen, 1999; Wild et al., 1997). Lp(a) is a lipoprotein particle similar to LDL with respect to its cholesterol and apolipoprotein B100 content, but it also contains an additional apolipoprotein termed apo(a) (Lippi and Guidi, 1999; Nielsen, 1999). Lp(a) concentrations have been reported by some investigators to be increased after the consumption of diets enriched in hydrogenated fat/*trans* fatty acids (Tables 8-9, 8-10, and 8-11) (Almendingen et al., 1995; Aro et al., 1997; Lichtenstein et al., 1999; Mensink et al., 1992; Nestel et al., 1992b; Sundram et al., 1997), but not by all (Chisholm et al., 1996; Judd et al., 1998; Lichtenstein et al., 1993; Louheranta et al., 1999; Müller et al., 1998). The magnitude of the mean increases in Lp(a) concentrations reported to date that is associated with *trans* fatty acid intake for the most part would not be predicted to have a physiologically significant effect on cardiovascular disease risk. However, an unresolved issue at this time is the potential effect of relatively high levels of *trans* fatty acids in individuals with initially high concentrations of Lp(a).

Hemostatic Factors. The effect of *trans* fatty acids on hemostatic factors has been assessed by a number of investigators (Almendingen et al., 1996; Mutanen and Aro, 1997; Sanders et al., 2000; Turpeinen et al., 1998; Wood et al., 1993b) (Table 8-12). In general, these researchers have concluded that hydrogenated fat/*trans* fatty acids had little effect on a variety of hemostatic variables. Similarly, Müller and colleagues (1998) reported that hemostatic variables were unaffected by the substitution of a vegetable oil-based margarine relatively high in saturated fatty acids when compared with a hydrogenated fish oil-based margarine.

Susceptibility of LDL to Oxidation. Hydrogenated fat/*trans* fatty acids have consistently been reported to have little effect on the susceptibility of LDL to oxidation (Cuchel et al., 1996; Halvorsen et al., 1996; Nestel et al., 1992b; Sørensen et al., 1998) (Table 8-12).

Blood Pressure. A few reports addressed the issue of *trans* fatty acid intake and blood pressure (Mensink et al., 1991; Zock et al., 1993) (Table 8-12). The authors concluded that consumption of diets high in saturated, mono-unsaturated, or *trans* fatty acids resulted in similar diastolic and systolic blood pressures.

CHD. Similar to saturated fatty acids, there is a positive linear trend between *trans* fatty acid intake and LDL cholesterol concentrations (Judd et al., 1994; Lichtenstein et al., 1999; Zock and Katan, 1992). Some evidence also suggests that *trans* fatty acids result in lower HDL cholesterol concentrations (Table 8-13). Hence, the net result is a higher total cholesterol or LDL cholesterol:HDL cholesterol ratio (Judd et al., 1994; Lichtenstein et al., 1999; Zock and Katan, 1992). This finding, combined with data from prospective cohort studies (Ascherio et al., 1996; Gillman et al., 1997; Hu et al., 1997; Pietinen et al., 1997; Willett et al., 1993) (Table 8-13), has led to the concern that dietary *trans* fatty acids are more deleterious with respect to CHD than saturated fatty acids (Ascherio et al., 1999).

Summary

Similar to saturated fatty acids, there is a positive linear trend between *trans* fatty acid intake and LDL cholesterol concentration, and therefore increased risk of CHD. A UL is not set for *trans* fatty acids because any incremental increase in *trans* fatty acid intake increases CHD risk. Because *trans* fatty acids are unavoidable in ordinary, nonvegan diets, consuming 0 percent of energy would require significant changes in patterns of dietary intake. Such adjustments may introduce undesirable effects (e.g., elimination of commercially prepared foods and dairy products and meats that

contain *trans* fatty acids may result in inadequate intakes of protein and certain micronutrients) and unknown and unquantifiable health risks. It is possible to consume a diet low in *trans* fatty acids by following the dietary guidance provided in Chapter 11.

RESEARCH RECOMMENDATIONS

Total Fat

- Studies are needed that examine the effects of alterations in the level of total fat in the context of a low saturated fatty acid diet on blood lipid concentrations and glucose-insulin homeostasis in individuals with defined metabolic syndromes, such as type 1 and type 2 diabetes.
- Randomized and blinded long-term (greater than 1 year) studies are needed on the effect of dietary fat versus carbohydrate on body fatness.

Saturated Fatty Acids

- Further examination of intakes at which significant risk of chronic diseases can occur is needed.
- Data that examine the indicators for and risk of chronic disease at low levels of saturated fatty acid intake are necessary.

Cis-Monounsaturated Fatty Acids

- Information is needed to assess energy balance in free-living individuals who have implemented a diet high in monounsaturated fatty acids versus a diet lower in monounsaturated fatty acids (and higher in carbohydrate).
- Additional information is needed on the effects of alterations in the level of monounsaturated fatty acid in the context of a low saturated fatty acid diet on blood lipid concentrations and glucose-insulin homeostasis in individuals with defined metabolic syndromes, such as type 1 and type 2 diabetes.
- Studies are needed to evaluate cardiovascular disease risk status and risk of other chronic diseases in individuals consuming a high monounsaturated fatty acid diet versus a diet lower in monounsaturated fatty acids (and higher in carbohydrate).
- An evaluation of the nutritional adequacy and nutrient profile of free-living individuals following a self-selected high monounsaturated fatty acid diet is necessary.
- Studies that assess the effects of a high monounsaturated fatty acid diet on endothelial function and atherogenesis are needed.

TABLE 8-12 *Trans* Fatty Acid (TFA) Intake and Blood Clotting, Low Density Lipoprotein (LDL) Oxidation, and Blood Pressure

Reference	Study Population	Diet ^a	TFA (% of energy)
Clotting			
Wood et al., 1993b	29 men, 30–60 y	6-wk crossover, 37% fat	
		Butter	0.2
		Crude palm oil	0
		Margarine	3.0
		Refined palm oil	0
		Refined palm+sunflower	0
		Sunflower oil	0
Almendingen et al., 1996	31 men, avg 27 y	3-wk crossover, 33–36% fat	
		PHSO	8.5
		PHFO	8.0
		Butter	0.9
Mutanen and Aro, 1997	80 men and women, 20–52 y	5-wk crossover to 1 of 2 diets, 33–34% fat	
		High 18:0	0.4
		High TFA	8.7
Turpeinen et al., 1998	80 men and women, 20–52 y	5-wk crossover to 1 of 2 diets, 32–34% fat	
		18:0	0.4
		TFA	8.7
Sanders et al., 2000	16 men and women, 18–32 y	1 test-meal crossover, 7% or 65% fat	
		18:1	0.1
		18:1 trans	24.7
		18:0	0
		16:0	0.2
		MCT	0
		Low fat	0
Oxidation			
Cuchel et al., 1996	14 men and women, 44–78 y	32-d crossover, 30% fat	
		Corn oil	0.44
		Corn oil+margarine	4.16

Results ^b		Comments
TxB ₂ (pg/mL)	6-keto-PGF _{1α} (pg/mL)	
35	89	
41	94	
40	86	
40	87	
36	100	
62	95	
Fibrinogen (g/L)	PAI-1 activity (units/mL)	For PHSO, greater PAI-1 activity than PHFO or butter
3.0	13.5	Increased fibrinogen with butter diet
2.9	10.7	No significant difference in factor VII, fibrinogen peptide A, β-thromboglobulin, or tissue plasminogen activator
3.1	8.8	
Fibrinogen (g/L)		No marked difference in factor VII coagulation activity, tissue type plasminogen activity, or PAI-1 activity
3.62		
3.61		
		No difference in TxB ₂ production or ADP-induced platelet aggregation in vitro
		Significant increase in collagen-induced aggregation with 18:0 diet
FVII _c (% standard)	FVII _a (ng/mL)	No significant differences in factor VII coagulation activity; factor VII-activated concentrations were significantly higher with 18:1, 18:1 trans, 18:0, and 16:0 diets
124	2.7	
122	1.9	
114	1.9	
112	2.1	
112	1.5	
99	1.4	
		No difference in susceptibility to LDL oxidation

continued

TABLE 8-12 Continued

Reference	Study Population	Diet ^a	TFA (% of energy)
Halvorsen et al., 1996	29 men, 21–46 y	19-d crossover, 33–36% fat	
		Butter	0.9
		PHSO	8.5
		PHFO	8.0
Sørensen et al., 1998	47 men, 29–60 y	4 wk, consumed 30 g/d of 1 of 2 margarines	<u>mol % of fat</u>
		Sunflower oil	0.79
		Fish oil, enriched	0.98
Blood pressure			
Mensink et al., 1991	59 men and women, 19–57 y, normotensive	3-wk crossover, 39–40% fat	
		18:1	0
		TFA	10.9
		SFA	1.8
Zock et al., 1993	55 men and women, 19–49 y	3-wk crossover, 40–43% fat	
		18:2	0.1
		18:0	0.3
		TFA	7.7

^a PHSO = partially hydrogenated soybean oil, PHFO = partially hydrogenated fish oil, MCT = medium-chain triacylglycerol, SFA = saturated fatty acid.

n-6 Polyunsaturated Fatty Acids

- In metabolic and large observational studies, comparison should be made of the benefits of α -linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) across a range of *n-6* polyunsaturated fatty acid intakes.

- Using good biomarkers for low density lipoprotein oxidation and cancer susceptibility, assessments are needed of the potential adverse effects of diets at levels of *n-6* polyunsaturated fatty acids greater than 10 percent of energy.

- Studies that assess the effects of a high *n-6* polyunsaturated fatty acid diet on markers of endothelial function and inflammation are needed.

Results ^b		Comments
Dienes (nmol/mg <u>LDL</u>)	Formation rate (nmol/mg <u>LDL × min</u>)	No significant differences in conjugated dienes, lipid peroxides, uptake by macrophages, or electrophoretic mobility of LDL TFA does not alter susceptibility to LDL oxidation
1,020	10	
1,034	10	
1,107	10	
Dienes (<u>nmol/g</u>)	Oxidation rate (nmol/mg × <u>min</u>)	Fish oil consumption compared with sunflower oil margarine had no effect on LDL size and led to minor changes in LDL oxidation resistance
445	10.4	
468	10.2	
		No effect of TFA intake on blood pressure
<u>SBP (mmHg)</u>	<u>DBP (mmHg)</u>	
113	66	
112	67	
112	67	
		No effect of TFA intake on blood pressure
<u>SBP (mmHg)</u>	<u>SBP (mmHg)</u>	
114	68	
113	70	
113	69	

^b TxB₂ = thromboxane B₂, 6-keto-PGF_{1α} = 6-keto-prostaglandin F_{1α}, PAI-1 = plasminogen activator inhibitor type 1, FVII_c = factor VII coagulant activity, FVII_a = factor VII activated, SBP = systolic blood pressure, DBP = diastolic blood pressure.

- Further research is needed to address the potentially important relationships between the amount of *n*-3 and *n*-6 fatty acids and glucose tolerance suggested by studies of fatty acid composition in affected individuals.

n-3 Polyunsaturated Fatty Acids

- Randomized clinical trials are needed of EPA+DHA, EPA, and DHA to evaluate their impact on cancer (i.e., colon, breast, prostate). The use of biomarkers for cancer susceptibility may expedite such studies.

TABLE 8-13 Dietary *Trans* Fatty Acids (TFA): Epidemiological Studies

Reference	Study Design ^a	Dietary and Other Information
<i>Lipoprotein concentration</i>		
Siguel and Lerman, 1993	47 CAD cases 56 controls Case-control	No dietary intake information
<i>Coronary heart disease (CHD)</i>		
Hudgins et al., 1991	76 men, 23–78 y Cross-sectional	No dietary intake information
Troisi et al., 1992	748 men, 43–85 y Cross-sectional	Food frequency questionnaire, multivariate analysis
Willett et al., 1993	Women, 431 CHD cases Cohort, 8-y follow-up	Food frequency questionnaire, multivariate analysis
Ascherio et al., 1994	239 MI cases 282 controls Case-control	Food frequency questionnaire, multivariate analysis
Kromhout et al., 1995	12,763 men, 40–59 y Cohort, 25-y follow-up	Weighed food record
Ascherio et al., 1996	43,757 men, 40–75 y Cohort, 6-y follow-up	Food frequency questionnaire, multivariate analysis

Results ^b			Comments ^c
<u>Plasma</u>	<u>Case</u>	<u>Control</u>	TFA negatively associated with HDL TFA positively associated with LDL and TAG
TFA (%)	1.38	1.11	
HDL (mmol/L)	0.88	1.34	
LDL (mmol/L)	3.78	2.97	
TAG (mmol/L)	1.78	0.97	
Total TFA in adipose tissue was 4.4% of total fatty acids			Total TFA content in adipose tissue was not significantly related to risk factors of CHD (e.g., age, BMI, LDL, cholesterol, blood pressure)
TFA intake was directly related to total ($r = 0.07$, $P = 0.04$) and LDL ($r = 0.09$, $P = 0.01$) cholesterol			An increased TFA intake from 2.1 to 4.9 g/d increased the risk of MI by 27%
<u>TFA intake</u> <u>(% energy)</u>	<u>RR of CHD</u>		Positive association with TFA intake and risk of CHD
1.3	1.0		
1.8	1.4		
2.2	1.25		
2.6	1.55		
3.2	1.8		
<u>TFA intake</u> <u>(g/d)</u>	<u>RR of MI</u>		Positive association of TFA intake and risk of myocardial infarction
1.69	1.0		
2.48	0.73		
3.35	1.24		
4.52	1.63		
6.51	2.28		
Correlation between 18:1 <i>trans</i> intake and CHD mortality is 0.78 ($p < 0.001$)			
<u>TFA intake</u> <u>(g/d)</u>	<u>RR of MI</u>		TFA intake directly associated with risk of MI
1.5	1.0		
2.2	1.20		
2.7	1.24		
3.3	1.27		
4.3	1.40		

continued

TABLE 8-13 Continued

Reference	Study Design ^a	Dietary and Other Information
Gillman et al., 1997	Men, 45–64 y 267 CHD cases Cohort, 21-y follow-up	24-h recall, multivariate analysis
Hu et al., 1997	Women, 34–59 y 939 MI cases Cohort, 14-y follow-up	Food frequency questionnaire, multivariate analysis
Pietinen et al., 1997	Smoking men, 50–69 y 1,399 coronary events 635 coronary deaths Cohort, 6.1-y follow-up	Food frequency questionnaire, multivariate analysis
Tavani et al., 1997	Women, 18–74 y 429 MI cases 866 controls Case-control	Questionnaire on selected indicator foods, multivariate analysis
<i>Cancer</i> Kohlmeier et al., 1997	Women, 50–74 y 291 breast cancer cases 407 controls Case-control	No diet information

Results ^b		Comments ^c
Margarine intake (tsp/d)	No. of events (/1,000) <u>Period 1</u> <u>Period 2</u>	RR for CHD for each increment of 1 tsp/d was 0.99 for follow-up period 1 and 1.12 for period 2 Modest risk of CHD with increasing margarine intake
0	77 65	
1-4	42 35	
≥ 5	18 30	
TFA intake (% energy)	<u>RR of MI</u>	RR for 2% increment in energy from TFA intake was 1.93
1.3	1.0	
1.7	1.07	
2.0	1.10	
2.4	1.13	
2.9	1.27	
TFA intake (g)	<u>RR of major coronary event</u>	Positive association between TFA intake and risk of coronary death
1.0	1.00	
1.7	1.10	
2.0	0.97	
2.7	1.07	
6.2	1.14	
TFA intake (g)	<u>RR of coronary death</u>	
1.0	1.00	
1.7	1.05	
2.0	1.12	
2.7	0.90	
6.2	1.39	
Margarine intakes	<u>RR of MI</u>	The association with margarine could explain about 6% of MI in this population
No or low	1.0	
Medium or high	1.5	
Adipose TFA concentration	<u>OR of breast cancer</u>	Risk for breast cancer is based on the relative concentration of TFA and PUFA
TFA	1.46	
TFA within lowest PUFA tertile	3.65	
TFA within highest PUFA tertile	0.97	

continued

TABLE 8-13 Continued

Reference	Study Design ^a	Dietary and Other Information
Tuyns et al., 1988	35–75 y 453 colon cancer cases 365 rectal cancer cases 2,851 controls Case-control	Dietary history

^a CAD = coronary artery disease, CHD = coronary heart disease, MI = myocardial infarction.

^b HDL = high density lipoprotein cholesterol, LDL = low density lipoprotein cholesterol, TAG = triacylglycerol, RR = relative risk, OR = odds ratio, PUFA = polyunsaturated fatty acid.

- Randomized clinical trials on the use of EPA+DHA, EPA, and DHA in treatment of inflammatory disorders (e.g., Crohn's disease, arthritis, psoriasis, asthma) and infections are needed.

- Studies of EPA+DHA, EPA, and DHA supplementation in the elderly to prevent degenerative diseases of the central nervous system and retina, such as dementia, age-related macular degeneration, and night blindness are needed.

Trans Fatty Acids

- A comprehensive database needs to be developed for the *trans* fatty acid content of the United States food supply; this database could then be used to determine the *trans* fatty acid intakes in different age and socio-economic groups.

- An assessment of major sources of *trans* fatty acids currently in the marketplace is needed, along with development of alternatives similar to that done for foods high in saturated fatty acids.

- Studies that distinguish *trans* fatty acid isomers from plants and animals with respect to the relative impact on blood lipid and lipoprotein concentrations are needed.

- In light of the wide variability of *trans* fatty acid intakes within food categories, the development of a biochemical marker for *trans* fatty acid intake, independent of self-reported intake data, is needed.

Results ^b	Comments ^c
	There was no increased risk of either cancers with increased consumption of margarine

^c BMI = body mass index.

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