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Developmental Toxicity Study of Vegetable Oil-Derived Stanol Fatty Acid Esters

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In a standard developmental toxicity study, a mixture of vegetable oil-derived stanol fatty acid esters was administered in the diet to groups of 28 mated female HsdCpb:WU Wistar rats at concentrations that provided 0, 1, 2.5, and 5% total stanols (equivalent to 0, 1.75, 4.38, and 8.76% plant stanol esters). Test diets were adjusted with rapeseed oil to maintain an equivalent caloric content of fatty acids at each of the treatment levels. The treatment period extended from day 0 to 21 of gestation. No compound-related toxicity or clinical effects were seen in any of the treated groups. No statistically significant differences were seen in body weights or body weight gain in the low- or mid-dose groups, although slight but statistically significant decreases in mean body weight relative to controls were seen at gestation days 7 and 14 in the high-dose group. The decreases in body weight in the high-dose group may be attributable to the virtual lack of absorption of the dietary stanols. Body weight gains were equivalent to controls throughout the study except for a statistically significant decrease seen only in the 0- to 7-day gestation period in the high-dose group. No significant effects were seen on food consumption in terms of g/rat/day, but a slight, statistically significant increase was seen in the mid-dose group during gestation days 7–14. A significant increase was seen in the high-dose group during the 7- to 21-day period of gestation. Reproductive performance was not affected by the treatment. There were no statistically significant differences in uterine weight, placental weight, fetal weight, number of fetuses, number of implantation sites, number of corpora lutea, and early/late resorptions between the treated and control groups. In addition, there was no biologically meaningful effect on fetal sex ratio. Visceral and skeletal examinations did not show any significant increases in the incidence of malformations, anomalies, or variations that were considered to be treatment related. Dietary plant (8.76% plant stanol esters) stanol esters at concentrations up to 5% total stanols were concluded to have no adverse effects on reproduction or development. © 1999 Academic Press

INTRODUCTION

Stanols are prepared from the hydrogenation of naturally occurring, mixed plant sterols that are found in wood (tall oil) and various vegetable oils. The conversion of the sterols to the corresponding stanols reduces their gastrointestinal absorption to negligible levels and increases their effectiveness at inhibiting cholesterol absorption (Heinemann *et al.*, 1991). For use in the commercial product, stanol esters are prepared by interesterification of stanols with the fatty acids of vegetable oils such as canola oil to improve their solubility in fats.

Stanol fatty acid ester mixtures derived from wood (tall oil) or vegetable oil have been used in Finland for some years in margarine spreads produced by the Raisio Group to help reduce serum total cholesterol and LDL cholesterol levels. The rationale for the use of plant stanol esters is that they can contribute to maintenance of cardiovascular health by reducing cholesterol absorption from the diet and from bile, with virtually no potential for absorption or having any undesirable pharmacological or toxicological effect.

The principal saturated stanol esters are mixed fatty acid esters of the 5α-stanols, sitostanol and campestanol. Sitostanol (24-ethylcholestan-3-ol, CAS No. 19466-47-8) is formed by the hydrogenation of the Δ^5-mono-unsaturated plant sterol, sitosterol (24-ethylcholest-5-en-3β-ol, CAS No. 83-46-5), and also by the complete hydrogenation of the $\Delta^{5,22}$-di-unsaturated plant sterol, stigmasterol (24-ethylcholest-5,22-dien-3β-ol, CAS No. 83-48-7), hence the alternative name "stigmastanol." Campestanol (24-methylcholestan-3β-ol) is formed by the hydrogenation of the Δ^5-mono-unsaturated plant sterol, campesterol (24-methylcholest-5-en-3β-ol, CAS No. 474-62-4).

The structures of sitostanol, of its immediate precursors sitosterol and stigmasterol, of campestanol, and of its immediate precursor campesterol are shown in Fig. 1. The structure of cholesterol, which differs from campesterol and sitosterol by the presence of a single methyl or ethyl group in the side chain, respectively, is shown for comparison.

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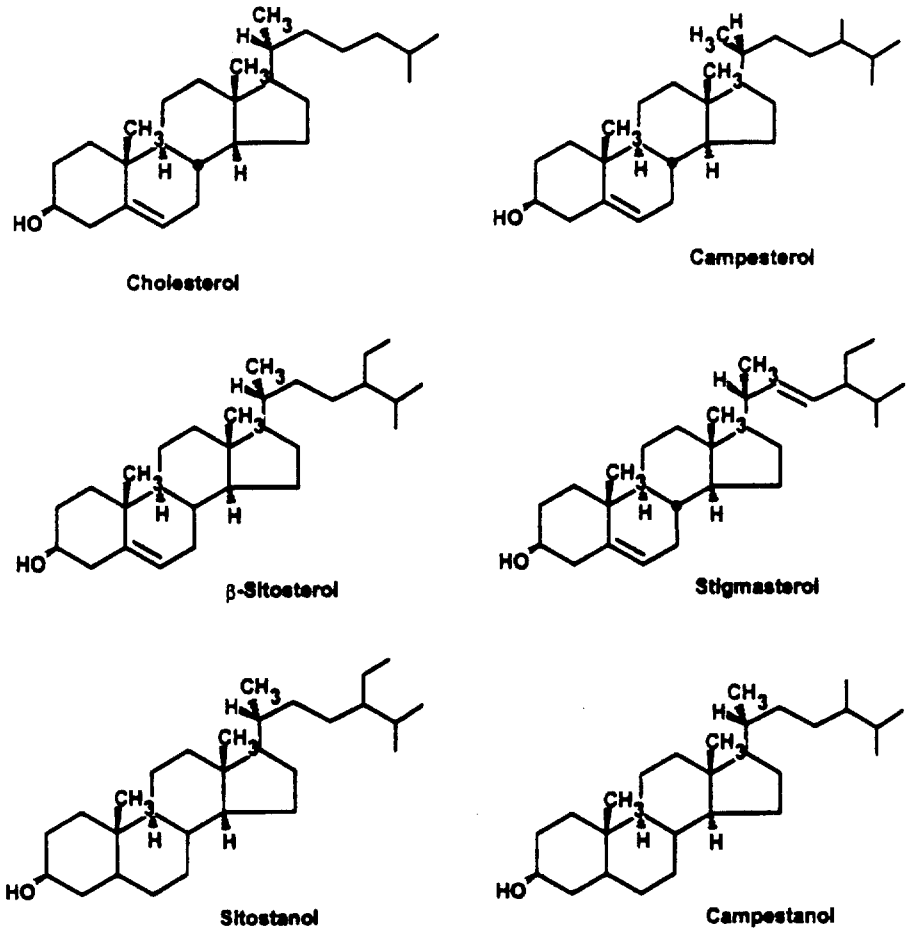

FIG. 1. Structure of cholesterol and some plant sterols.

There is growing interest in utilization of plant-derived sterols and stanols to help reduce serum cholesterol levels in humans. Cholesterol-lowering effects of plant-derived sterol and stanol mixtures were seen in studies with 22 subjects who were fed diets with or without 21.2 mg/kg body wt tall oil-derived sterols that contained 21% of the saturated sterol sitostanol (Jones *et al.*, 1998). The subjects were given either the supplemented or placebo diets over two 10-day periods in a randomized crossover study design. Total cholesterol and low-density lipoprotein cholesterol levels were significantly reduced ($P = 0.01$) in subjects given the plant-derived sterol/stanol mixtures versus the placebo group. Sitostanol levels were essentially undetectable in plasma, indicating that there is a low potential for absorption of plant stanols. A review of the physiological effects of plant sterols in human diets by Jones *et al.* (1997) concluded that plant sterols inhibit the absorption of cholesterol. However, there was a greater reduction of plasma cholesterol levels from consumption of the saturated plant stanols such as sitostanol in comparison to lesser effects produced by unsaturated plant sterols (e.g., sitosterol or campesterol). Results from a parallel, double-blind study with 55 hypercholesterolemic subjects by Hallikainen and Uusitupa (1999) confirmed the cholesterol-lowering effects noted by Jones *et al.* (1998) in dietary studies with margarine fortified with plant stanol esters. In groups that consumed stanol-ester-fortified margarine for 8 weeks, researchers observed statistically significant decreases in comparison to controls for serum total cholesterol from 8.1 to 10.6% and in LDL cholesterol from 8.6 to 13.7% with two different plant stanol esters. They concluded that lowfat plant stanol-ester-foritified margarines are effective cholesterol-lowering products that produce clinically significant reductions in serum cholesterol that were greater than obtained by a lowfat diet alone.

As part of a comprehensive safety assessment of plant stanol fatty acid esters, pregnant rats were fed diets containing increasing amounts of stanol esters for determination of potential effects upon maternal and developmental toxicity.

MATERIALS AND METHODS

Animals

Male and virgin female SPF Wistar outbred rats (Hsd/Cpb:WU) were obtained from Harlan Netherlands B.V.,

Horst, The Netherlands. Rats were quarantined and acclimated for a total of 11 days in groups of 4 rats/sex/cage. The animal room was maintained at a temperature at 22 ± 3°C, a relative humidity range of 47–88%, and a 12-h light/dark cycle. At the start of mating the females were at least 12 weeks of age and their mean body weights ranged between approximately 241 and 248 g.

Diets and Test Materials

Feed and water were supplied *ad libitum* throughout the study and food consumption was determined weekly. During the acclimatization period rats were fed a defined powdered diet obtained from Special Diets Services, Witham, England. The plant stanol ester product was fed to the rats in the same diet that was supplemented with rapeseed oil to equalize the energy contribution from the fatty acids in the diets with varying amounts of the stanol esters. A total of 3.68% total fatty acids from rapeseed oil and the test substance was maintained in all control and test diets with the assumption that both the test substance-derived fatty acids and rapeseed oil provide 9 kcal/g. The diets contained 0, 1, 2.5, or 5% total stanols (corresponding to 0, 1.75, 4.38, and 8.76% of the stanol ester test material. All test diets were maintained refrigerated at 2–10°C.

The fortified test diets were analyzed for stability, content, and homogeneity of the test substance. The analytical procedure involved acid hydrolysis, extraction into petroleum ether, alkaline hydrolysis of the dried ether extracts, derivatization, and quantification by GC/FID. Recovery of the stanol from fortified diets varied within a range of 96.7 to 97.9% for the three test diets. The test substance was found to be stable in the diet for at least 7 days at room temperature and for at least 39 days refrigerated at 2–10°C. Analyses showed that the stanol ester was homogeneously distributed in the diets with a coefficient of variation of <4% for five samples of each diet. The actual versus intended content of total stanols in the three test diets varied from 91.8 to 97.5%. Table 1 summarizes the nutrient content of the diets used during the study.

The test substance was a grayish-white waxy solid consisting of stanol fatty acid esters derived from vegetable oil precursors obtained from Raision Tehaat OY AB, Raisio, Finland. The sample identified as Sito-70 stanol ester contained 57.08% total stanols/100 g fat (68% sitostanol, 30% campestanol, 2% unsaturated sterol), 41.96% fatty acids, and 2% unsaturated sterols and unknowns. Approximately 93.4% of the esterified groups was C-18 fatty acids, with 3.6% C-16, 2.1% C-20, and 0.9% other fatty acid esters. The purity of the test sample was approximately 99%.

Experimental Design

The study was performed according to OECD Guideline 414 and was in compliance with OECD Principles of Good Laboratory Practices by TNO Nutrition and Food Research Institute, Zeist, The Netherlands. For mating, two females were housed with one male until vaginal examinations each morning revealed that copulation had occurred by observing the presence of sperm cells in a vaginal smear. The day that observation was made was considered to be gestation day 0 and females were housed individually for the remainder of gestation. The mated females were distributed over the control and treatment groups such that the rats that became pregnant on the same day were equally distributed over all groups. Females impregnated by the same male were placed in different treatment groups.

TABLE 1
Compositional Analysis of Rat Chow

Nutrient	Found analysis[a]
Moisture	9.6–11.6%
Crude fat	4.1–4.8%
Crude protein	22.5–23.5%
Crude fiber	4.2–4.9%
Ash	6.4–6.8%
Calcium	0.95–1.02%
Phosphorus	0.77–0.80%
Sodium	0.29–0.31%
Chloride	0.47–0.56%
Potassium	0.71–0.80%
Magnesium	0.22–0.23%
Iron	145–184 mg/kg
Copper	15–19 mg/kg
Manganese	68–70 mg/kg
Zinc	60–68 mg/kg
Vitamin A	9.8–16.3 mg/kg
Vitamin E	55–109 mg/kg

[a] Values are ranges for two batches of rat chow.

The test article was given in the diet to 28 rats per dose group from day 0 to 21 of gestation. All rats were examined twice daily and once on weekends and holidays for pharmacologic or toxicologic signs and for mortality. Body weights of the pregnant rats were recorded on days 0, 7, 14, and 21 of gestation. The quantity of food consumed by each animal was measured during gestation days 0–7, 7–14, and 14–21. Rats were sacrificed on day 21 of gestation following ether anesthesia and examined for gross abnormalities. The uteri and ovaries of all females were examined for the number of corporal lutea, number of implantation sites, number of early and late resorptions, number of live and dead fetuses, sex of fetuses, number of grossly visible malformed fetuses, and fetuses with external abnormalities. Weights were recorded for ovaries, uteri containing the placentas and fetuses, uteri empty, fetuses, and placentas.

Each fetus was examined for external anomalies and for sex. Half of the fetuses in each litter were fixed in Bouin's fixative and subsequently examined for soft-tissue anomalies according to a modification of a method described by Barrow and Taylor (1969). The

TABLE 2
Body Weight, Body Weight Gain, and Food Consumption of Female Wistar Rats Fed Diets Containing up to 5% Plant Stanols during Days 0–21 of Gestation

Parameter[a] (day/period)	Dietary percentage of plant stanols (% stanol esters)			
	0	1 (1.75)	2.5 (4.38)	5 (8.76)
Number of pregnant rats	25	28	26	25 (24[b])
Mean body weight (g)				
Day				
0	247.56 ± 2.45	246.07 ± 1.91	245.28 ± 2.06	240.97 ± 1.94[b]
7	279.26 ± 2.70	277.71 ± 2.01	275.92 ± 2.28	270.07 ± 2.63*
14	310.07 ± 2.77	309.03 ± 2.20	306.45 ± 2.35	300.30 ± 3.08*
21	330.83 ± 3.04	331.08 ± 2.90	327.05 ± 3.32	322.82 ± 4.01
Mean body weight gain (g)				
Days				
0–7	31.70 ± 1.31	31.65 ± 0.94	30.65 ± 1.07	27.71 ± 1.12[b]*
7–14	30.81 ± 1.36	31.32 ± 0.66	30.52 ± 0.92	30.23 ± 0.91
14–21	20.76 ± 1.55	22.05 ± 1.86	20.61 ± 1.82	22.52 ± 2.80
Mean food intake (g/kg body wt/day)				
Days				
0–7	69.48 ± 0.94	69.39 ± 0.84	70.92 ± 0.91	70.42 ± 0.80[b]
7–14	66.17 ± 0.74	67.62 ± 0.67	69.20 ± 0.87*	71.14 ± 0.77**
14–21	43.64 ± 0.91	44.66 ± 1.05	45.72 ± 1.10	48.42 ± 1.67*
Test substance intake (g total stanols/kg body wt/day)[c]				
Days				
0–7	—	0.7 (1.2)[c]	1.8 (3.1)	3.5 (6.2)
7–14	—	0.7 (1.2)	1.7 (3.0)	3.6 (6.2)
14–21	—	0.4 (0.7)	1.4 (2.0)	2.4 (4.2)

[a] Values are means ± SE.
[b] One animal weight was not used in calculations.
[c] Values in parentheses are for intake expressed as g stanol ester product/kg body wt/day.
* $P < 0.05$, ** $P < 0.001$.

other half of the fetuses/litter were fixed in 70% ethanol, partially eviscerated, and then cleared in potassium hydroxide, stained with Alizarin Red S, and examined for skeletal anomalies.

Statistical Analysis

Statistical evaluations of the equality of means for body weights, body weight gain, organ weights, and food consumption data were performed by a one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test. Clinical findings and fetopathological data were evaluated by Fisher's exact probability test. Differences in the number of pregnant females and females with live fetuses were evaluated using Fisher's exact probability test. Numbers of corpora lutea, implantations, live and dead fetuses, and early and late resorptions were evaluated by Kruskal–Wallis nonparametric analysis followed by the Mann–Whitney *U* test.

RESULTS

Maternal Observations

Diets containing up to 5% stanols (8.76% plant stanol esters) were well tolerated. Daily clinical observations during the gestation period did not reveal any adverse findings in the rats' appearance, general condition, or behavior in any of the groups. No mortalities occurred in the control or stanol ester-treated groups. Mean body weights and body weight gain of pregnant females during gestation were not significantly different from the control values throughout the study in the groups fed 1 and 2.5% total stanols. In the group fed 5% stanols a significant reduction in mean body weight relative to controls was observed at gestation days 7 and 14 but not at day 21 (Table 2). Body weight gains were not significantly different from control values for any group throughout gestation except for a small but statistically significant decrease at days 0–7 for the high-dose group (Table 2).

Food consumption (expressed as g/animal/day) showed no significant differences from controls. A small but statistically significant increase in food consumption (expressed as g/kg body wt/day) was seen in the mid-dose group during gestation days 7–14 and in the high-dose group during gestations days 7–14 and 14–21. Intake of the test substance calculated from the food consumption data expressed as g total stanols/kg body wt/day showed no remarkable differences except for moderate decreases in the dose of the test substance

TABLE 3
Reproductive Performance of Female Wistar Rats Fed Diets Containing up to 5% of Plant Stanols during Days 0–21 of Gestation

Parameter	Dietary percentage of plant stanols (% stanol esters)			
	0	1 (1.75)	2.5 (4.38)	5 (8.76)
Females Mated	28	28	28	28
Delivered prematurely (*n*)	0	0	0	0
Pregnant at necropsy (*n*)	25	28	26	25
With live fetuses (*n*)	25	28	26	25
With no viable fetuses	0	0	0	0
Corpora lutea (*n*/rat)[a]	14.28 ± 0.35	14.14 ± 0.32	13.73 ± 0.34	13.36 ± 0.29
Implantation sites (*n*/rat)[a]	13.36 ± 0.37	13.11 ± 0.42	12.65 ± 0.35	12.28 ± 0.50
Preimplantation loss (%/rat)[a,b]	6.29 ± 1.62	7.57 ± 1.73	7.71 ± 1.58	8.40 ± 3.14
Live fetuses (*n*/rat)[a]	13.04 ± 0.36	12.75 ± 0.46	12.35 ± 0.36	12.12 ± 0.51
Postimplantation loss (%/rat)[a,c]	2.21 ± 0.97	2.83 ± 1.39	2.56 ± 0.80	1.30 ± 0.91
Dead fetuses (*n*/rat)	0	0	0	0
Resorptions total (*n*/rat)[a]	0.32 ± 0.14	0.36 ± 0.19	0.31 ± 0.09	0.16 ± 0.11
Resorptions early (*n*/rat)[a]	0.32 ± 0.14	0.32 ± 0.19	0.31 ± 0.09	0.16 ± 0.11
Resorptions late (*n*)[a]	0.00	0.04 ± 0.04	0.00	0.00
Total number of fetuses (*n*)	326	357	321	303
Male fetuses (%)	57	47*	49	56
Female fetuses (%)	43	53*	51	44
Gravid uterus (g)[a]	76.95 ± 1.72	75.29 ± 2.05	73.40 ± 1.77	71.92 ± 2.31
Empty uterus (g)[a]	5.12 ± 0.16	4.91 ± 0.15	4.85 ± 0.14	4.81 ± 0.14
Ovaries (g)[a]	0.11 ± 0.006	0.11 ± 0.004	0.11 ± 0.004	0.11 ± 0.004
Placenta weight: All fetuses (g)[a]	0.54 ± 0.01	0.53 ± 0.01	0.56 ± 0.01	0.55 ± 0.01
Body weight of viable fetuses (g)[a]	4.29 ± 0.06	4.33 ± 0.08	4.37 ± 0.07	4.34 ± 0.09
Male fetuses (g)[a]	4.38 ± 0.06	4.43 ± 0.09	4.49 ± 0.07	4.45 ± 0.10
Female fetuses (g)[a]	4.18 ± 0.06	4.24 ± 0.08	4.26 ± 0.07	4.21 ± 0.08

[a] Values are means ± SE.
[b] Preimplantation loss = [number of corpora lutea − number of implantations sites)/number of corpora lutea] × 100.
[c] Postimplantation loss = [number of implantation site − number of live fetuses)/ number of implantation sites] × 100.
* $P < 0.05$, Fisher's exact test.

ingested at gestation days 14–21 in all three dose groups (Table 2).

All of the females were sacrificed on day 21 of gestation. At caesarian section, 25, 28, 26, and 25 females of the total of 28 mated rats in the control and low-, mid-, and high-dose groups were pregnant (Table 3). Gross examinations of the maternal organs and tissues did not reveal any significant or treatment-related differences among the stanol-ester-treated and control groups.

Reproductive Performance

Reproductive performance data are summarized in Table 3. Pregnancy rates for all treatment groups were the same or slightly higher than in the controls. There were no statistically significant differences between the control group and groups given increasing amounts of stanol esters in the diet in the number of corpora lutea, number of implantations, number of live and dead fetuses, and number of early and late resorptions or in pre- and postimplantation loss. There were no statistical differences in the weights of the ovaries, gravid and empty uteri, or placentas in comparison to control and treated groups. The percentages of males and females were similar for all groups, although there was a slight but significant decrease in the number of male fetuses as well as a corresponding slight but significant increase in the number of female fetuses at the lowest dose level. This difference was not considered to be treatment related because sex ratios at higher doses were comparable to the control.

Fetal Examination

At caesarian section, a total of 1307 live fetuses were examined in 104 litters for external abnormalities. Mean fetal body weights categorized by sex and for combined sexes were not significantly different from control for any treatment group. Placental weights were similar to controls for all groups (Table 3). No differences in the number or type of external malformations were noted in fetuses in the control and treatment groups. In one female of the control group and two females of the mid-dose group, two placentas were fused but these findings were not statistically significant and were not considered treatment related.

No increases in visceral malformations were ob-

TABLE 4
Visceral Malformations, Anomalies, and Variations in Wistar Rats Fed Diets Containing up to 5% of Plant Stanols during Days 0–21 of Gestation

Parameter[a]	Dietary percentage of plant stanols (% stanol esters)			
	0	1 (1.75)	2.5 (4.38)	5 (8.76)
Fetuses (litters) examined	157 (25)	170 (28)	152 (26)	146 (25)
Malformations total	4 (2)	0 (0)	0 (0)	0 (0)
Brain: hydrocephaly	1 (1)	0 (0)	0 (0)	0 (0)
Kidneys: hydronephrosis	3 (1)	0 (0)	0 (0)	0 (0)
Anomalies total	30 (13)	28 (12)	7*** (5)*	8*** (5)*
Dilated esophagus	0 (0)	0 (0)	0 (0)	1 (1)
Dilated urinary bladder	24 (10)	24 (11)	4*** (3)*	6** (4)
Ureters, hydroureter	10 (5)	5 (2)	3 (2)	2* (2)
Variations total	26 (14)	32 (16)	23 (11)	15 (8)
Stomach distended	1 (1)	0 (0)	0 (0)	0 (0)
Stomach with hemorrhagic fluid	9 (6)	6 (4)	7 (5)	7 (5)
Kidney: Increased renal pelvic cavitation	13 (8)	20 (11)	12 (7)	5 (4)
Ureters: Bent	6 (5)	10 (6)	12 (7)	4 (4)

[a] Values are incidences in fetuses (litters).
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

served in the fetuses of groups fed with stanol esters (Table 4). The incidence of visceral anomalies showed a statistically significant decrease in the total number of fetuses with anomalies due to a decrease in the incidence of fetuses with dilated urinary bladders in the mid- and high-dose groups in comparison to the controls. There was also a dose-related decrease in the incidence of fetuses with hydroureter that was significantly lower than the control at the high-dose level. Although these results showed a dose-related decrease between the concentration of rapeseed oil in the diet and dilated urinary bladder and hydroureter, there are no reports in the literature that describe a relationship between fetal developmental abnormalities and high concentrations of rapeseed oil. Also, the incidence of dilated urinary bladder and hydroureter in the control and low-dose groups was outside the upper limits of the historical control range of the testing facility. The number and type of visceral variations seen were considered to be typical for rats and no significant differences were noted among the control and treated groups.

A total of 682 live fetuses from 104 litters were examined for skeletal malformations, abnormalities, and variations (Table 5). In 3 fetuses of 2 litters of the low-dose group, missing ribs were observed. No other skeletal malformations were observed in the control or higher dose groups. No significant increases in skeletal anomalies were observed in the stanol-ester-treated groups. Skeletal anomalies were observed in 1 fetus in the low-dose group that had wavy ribs, 1 fetus that had separated sternebrae, and 1 fetus that had a few ribs with reduced size. No significant differences were found in skeletal variations in any of the treated groups and the variations noted were considered to be typical observations in rat studies.

Variations in ossification were significantly greater than controls in the high-dose group that had a higher incidence of fetuses with incompletely ossified frontalis and extra caudal bodies. Significant differences were also seen in the mid-dose group for increased incidence of incompletely ossified frontalis and a decreased incidence of ossification of proximal phalanges of the front

TABLE 5
Skeletal Malformations, Anomalies, and Variations in Wistar Rats Fed Diets Containing up to 5% of Plant Stanols during Days 0–21 of Gestation

Parameter[a]	Dietary percentage of plant stanols (% stanol esters)			
	0	1 (1.75)	2.5 (4.38)	5 (8.76)
Fetuses (litters) examined	169 (25)	187 (28)	169 (26)	157 (25)
Skeletal malformations:				
Total	0 (0)	3 (2)	0 (0)	0 (0)
Ribs: Missing	0 (0)	3 (2)	0 (0)	0 (0)
Skeletal anomalies: Total	1 (1)	3 (2)	0 (0)	0 (0)
Ribs				
Two or more ribs wavy	0 (0)	1 (1)	0 (0)	0 (0)
Two or more reduced in size	0 (0)	1 (1)	0 (0)	0 (0)
Sternebrae: Separated	1 (1)	1 (1)	0 (0)	0 (0)
Skeletal variations: Total	41 (18)	58 (23)	51 (21)	43 (21)
Ribs: Accessory lumbar ribs	2 (1)	9 (6)	6 (5)	7 (5)
Sternebrae				
Irregular shape of one	12 (10)	17 (12)	16 (13)	18 (12)
Irregular shape of two or more	27 (13)	37 (19)	27 (14)	19 (12)
One supernumerary	1 (1)	0 (0)	5 (1)	1 (1)

[a] Values are incidences in fetuses (litters).

legs. The low-dose group had a reduced incidence of fetuses with an incompletely ossified interparietalis and incidence of five to eight proximal phalanges on the hind legs but an increased incidence of incompletely ossified 0–2 metatarsals. No statistically significant differences were noted in skeletal variations when the data were analyzed on a litter basis and the incidence of variations showed no apparent relationship with increasing dose. Because of the apparent lack of a treatment-related effect, the incidence in variations in ossification were not considered to be biologically significant and the data were not tabulated.

DISCUSSION

A diet containing up to 5% plant stanols (8.76% plant stanol fatty acid esters) (equivalent to a dietary intake of 2.4–3.5 g total stanols/kg body wt/day) was well tolerated by the pregnant rats. There were no mortalities or adverse effects on appearance, physical condition, or behavior during days 0–21 of gestation. No compound-related adverse toxicity was seen in any of the stanol-ester-treated rats and no significant increases were seen in the incidence of visceral or skeletal malformations, abnormalities, or variations. Statistically significant differences were noted in mean body weight relative to controls at the 0- to 7-day and 7- to 14-day period and in body weight gains during 0–7 days for the high-dose group. One explanation for these body weight effects could be that there was a decrease in caloric content of the diet from the levels of unabsorbable stanols at the highest dose. As the amount of stanol increased, concomitant with increasing dose, the amount of rapeseed oil decreased accordingly and the fatty acid esters in the product may not have been equivalent to the rapeseed oil fatty acids. The changes in body weight at the highest dose were relatively small, were transient in nature, and were not considered biologically meaningful as they were not seen in the 14- to 21-day terminal portion of the study.

CONCLUSION

No adverse treatment-related maternal or fetal developmental effects were produced following ingestion of a diet containing up to 8.76% plant stanol fatty acid esters. This diet provided up to 5% of total dietary stanols equivalent to 2.4–3.5 g stanols/kg body wt/day. No significant differences were seen in reproductive performance, maternal and fetal body weights, sex distribution, or visceral or skeletal malformations, anomalies, and variations. Vegetable oil-derived stanol fatty acid esters are concluded not to be developmental toxicants and did not produce any embryotoxic, fetotoxic, or teratogenic effects in Wistar rats under the conditions of this study.

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Review Article

BIOLOGICAL ACTIVITIES OF OXYSTEROLS

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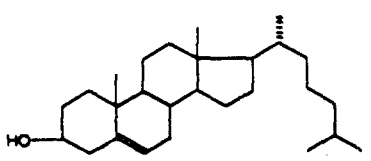
Abstract—Literature dealing with the biological activities of cholesterol autoxidation products and related oxysterols in vivo and in vitro published since the previous 1981 monograph is reviewed. Although several oxysterols are important cholesterol metabolites implicated in bile acids and steroid hormones biosynthesis, effects on cellular membranes and on specific enzyme systems as well as cytotoxic, atherogenic, mutagenic, and carcinogenic activities characterize oxysterols as a class. Circumstantial evidence implicates oxysterols of the human diet and those formed in vivo with human health disorders, but recent work also supports an hypothesis that some oxysterols be endogenous intracellular regulators of de novo sterol biosynthesis. The true physiological relevance, if any, of these matters has not been adduced.

Keywords—Oxysterols, Cholesterol oxidation products, Cholesterol autoxidation, Biological actions, Biological activities, Oxidized sterols

INTRODUCTION

The current focus on biologically active oxygen species and associated radicals is of crucial importance to understanding of life processes in an imposing oxygen environment. However, the present review does not dwell on active oxygen species and radical effects but on the biological effects of derivatives of their actions on tissue unsaturated lipids, specifically on cholesterol (cholest-5-en-3β-ol, *1*). The metabolic disposition and



1 Cholesterol

biological activities of product oxidized sterols, the oxysterols, are presently of concern with respect to human health matters. Related interest in biological actions of other oxidized unsaturated lipids is also current but is beyond the scope of the present review.

Cholesterol ubiquitously present in mammalian tissues is essential for formation and function of cellular membranes, is the obligate precursor of the bile acids and steroid hormones, and may have yet other functions in vivo. The relationship of cholesterol to human atherosclerosis evinces the importance of the sterol to human disease. Cholesterol is subject to oxidations by divers active oxygen species, yielding the biologically active oxysterols of present interest. Other sterols desmosterol (cholesta-5,24-dien-3β-ol), lanosterol (5α-lanosta-8,24-dien-3β-ol), 24-alkylsterols, etc. are also oxidizied to biologically active oxysterols, although much less is known about these.

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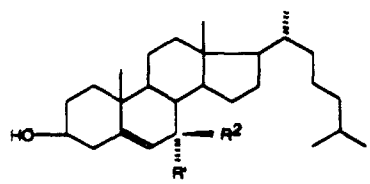
Oxysterols are uniformly toxic in a wide variety of in vivo tests and in vitro bioassays. These matters were reviewed in detail in 1981,[1] and subsequent treatments have appeared.[2-4] Interests have continued at a steady pace since 1981, with about 50 items per year dealing with chemistry and biochemistry[2] and an equal number with biological activities. The present review attempts to cover material dealing with the biological activities of oxysterols described since the 1981 monographic treatment, including items inadvertantly not treated therein. It is presumed that some familiarity with the 1981 monograph coverage be had by the reader.

The oxysterols included in this review are those that retain the sterol side-chain in good part, thus with longer side-chains than the C_{21}-steroids. Accordingly, hormonally active cholesterol autoxidation products such as dehydroepiandrosterone are not reviewed here. Oxysterols receiving the greatest attention are those readily available commercially: cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol, *2*), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol, *3*), 3β-hydroxycholest-5-en-7-one (7-ketocholesterol, *4*), 5,6α-epoxy-5α-cholestan-3β-ol (cholesterol 5α,6α-epoxide, *5*), 5α-cholestane-3β,5,6β-triol *6*), and cholest-5-ene-3β,25-diol (25-hydroxycholesterol, *7*). Also 5,6β-epoxy-5β-cholestan-3β-ol (cholesterol 5β,6β-epoxide, *8*), cholest-4-en-3-one (*9*), (20S)-cholest-5-ene-3β,20-diol (20-hydroxycholesterol, *10*), (25R)-cholest-5-ene-3β,26-diol (26-hydroxycholesterol, *11*), and other common oxysterols have been examined, as have also oxysterols from special tissue sources and from syntheses devised for testing as cytotoxic agents against malignant tumor cells or for inhibition of de novo sterol biosynthesis to achieve lowering of plasma sterol levels.

Oxysterols are reported to influence such vital matters as de novo sterol biosynthesis, membrane function, DNA synthesis, cell growth and proliferation, and aortal atherosclerosis also associated with the parent cholesterol. Thus, there arises a natural concern over



2 R^1 = OH, R^2 = H
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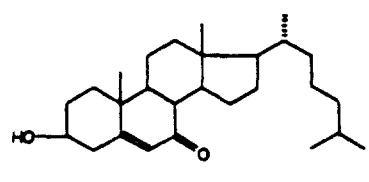

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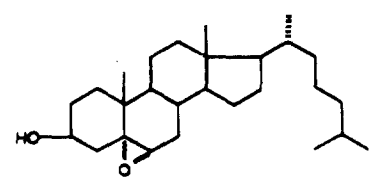
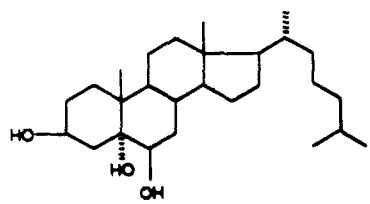


5 Cholesterol 5α, 6α - epoxide
8 Cholesterol 5β, 6β - epoxide



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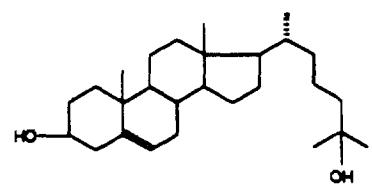

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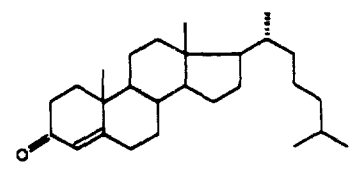

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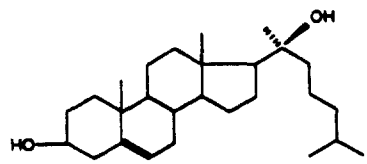

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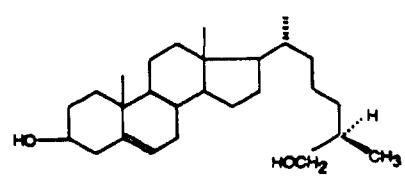

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whether cholesterol or oxysterol (or both) be the active agent. In bioassays of cholesterol dispersed in media exposed to air there is also question about whether effects be those of the parent sterol or of traces of highly active oxysterol inadvertantly formed before or during bioassay.

Although oxysterols with biological activities are emphasized here, it is also important to note that the biological activity of a parent sterol may be different depending on whether associated oxysterols be present or absent. This point is of importance in review of the relationship of dietary cholesterol to human atherosclerosis, where the presence of traces of oxysterols may in fact be the atherogenic or angiotoxic agents. The point also holds for Vitamin D_3 (cholecalciferol, (5Z,7E)- 9,10-secocholesta- 5,7,10(19)- trien-3β-ol) also highly susceptible to autoxidations, where Vitamin D_3 preparations stabilized against oxidation during manufacture retain greater potency and associated toxicity![5]

In view of the uncertainties posed, one is drawn directly to a range of questions which if answered might lead to an improved understanding of the topic. Among the questions are:

1. What kinds of biological activities characterize oxysterols? Are oxysterols uniformly toxic?

The present review of recent progress indicates that oxysterols as a class are toxic agents at levels tested. However, whether oxysterols toxicities pose harmful or beneficial effects in vivo is uncertain. Moreover, an hypothesis suggesting that certain endogenously formed oxysterols serve as intracellular regulatory agents suppressing de novo sterol biosynthesis by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, if so in vivo provides another importance for some oxysterols.

2. Are oxysterols present in the diet or are they formed in vivo? If formed in vivo, by what processes?

Oxysterols are indeed present at low levels (part-per-million to part-per-billion) in cholesterol-rich processed foods[2,6-10] and at similar levels in freshly drawn human blood;[11-16] both dietary and endogenous metabolism origins exist. It is also possible that diet stimulate in vivo creation of oxysterols, a matter to be carefully considered. Thus, elevated levels of the 5,6-epoxides *5* and *8* and of the 3β,5α,6β-triol *6* were found in liver and plasma of cholesterol fed rabbits.[17] Chronic ingestion of dietary oxysterols, even at low levels, may pose an avoidable toxic burden, and as potentially toxic components of foods, oxysterols have attracted the attention of the processed foods industry and the U.S. Departments of Agriculture and Defense.

Anent modes of oxysterol formation, in addition to the well-known radical autoxidation origin of oxysterols from cholesterol exposed to ground-state (triplet) dioxygen ($^3O_2$) of the air, oxidized sterols are also formed from cholesterol exposed to other active oxygen species, including electronically excited (singlet) dioxygen ($^1O_2$), peroxide (O_2^-, HOO^-, H_2O_2), hydroxyl radical ($HO\cdot$), dioxygen cation (O_2^+), and ozone (O_3). Of the well-known active oxygen species, only superoxide (O_2^-, $HOO\cdot$) fails to oxidize cholesterol.[18-20] Other environmental oxidants also react with tissue cholesterol; for instance, atmospheric NO_2 forms cholesterol 3β-nitrite and 5,6-epoxides *5* and *8* in exposed skin or lung tissues.[21-24]

Oxysterols have several in vivo origins, including tissue cholesterol enzymic hydroxylations at the 7α-, $20\alpha_R$-, $22\beta_R$-, 23-, $24\beta_R$, 25-, 26-, and 27-sites, 5α,6α-epoxidation, and 3β-hydroxysteroid dehydrogenation, all yielding oxysterols. Additionally, intravascular origins exist; for example, the 3β,7α-diol *2* formed in and secreted from liver (in bile acid biosynthesis)[25] may also be formed in human plasma by leukocytes which possess a cholesterol 7α-hydroxylase![26] Moreover, hepatic lipid peroxidation of cholesterol established in vitro and yielding the same oxysterols as radical autoxidation may also occur in vivo, potentially supplemented by intravascular lipid peroxidations by leukocytes.[11,12]

These several processes ensure that oxysterols be present at basal levels in human plasma or tissues, perhaps subject to fluctuations influenced by diet or health state.

3. Are ingested dietary oxysterols absorbed from the gastrointestinal tract?

Evidence suggests that dietary oxysterols are absorbed from the gastrointestinal tract rapidly.[27-34] Some absorption of (25R)-3β,26-diol *11* 3β,26-disulfate administered per os in human neonates is also indicated.[35]

4. Are ingested or endogenously formed oxysterols detoxified by protective metabolism for harmless excretion?

Oxysterols are clearly subject to esterifications in vivo and in vitro, but it is uncertain whether these transformations act as transporting or detoxifying processes. The cholesterol 5,6-epoxides are subject to enzymic hydrolysis, but the 3β,5α,6β-triol *6* metabolite

is more toxic. The 5α,6α-epoxide *5* fed rats is also metabolized to bile acids, presumably benignly.[28]

The cholesterol metabolites 3β,7α-diol *2*, 3β,25-diol *7*, and 3β,26-diol *11* implicated in hepatic biosynthesis of bile acids, the 3β,20-diol *10* and (22R)-cholest-5-ene-3β,22-diol implicated in steroid hormone biosynthesis, the enone *9* implicated in cholesterol reduction to stanols, and (24S)-cholest-5-ene-3β,24-diol (cerebrosterol) of central nervous tissue obviously have metabolic fates of other sorts.

5. Do oxysterol compositions of tissues and plasma or their levels fluctuate with diet or health state?

The matter of fluctuating levels of plasma oxysterols is as yet uncertain, but differences between plasma levels of the 3β,26-diol *11* in younger people versus older patients with cardiovascular disease[36] and between levels of the 3β,7-diols *2* and *3* and the 3β,26-diol *11* in normals versus patients with cerebrotendinous xanthomatosis[37-39] suggest such possibility. High levels of the 5α,6α-epoxide *5* in blood of hypercholesterolemic patients is also indicated by older analyses.[40] Fluctuations associated with nutrition or diet have not been investigated.

The question of fluctuating oxysterols blood levels cannot now be posed so naively, as oxysterols appear to be partitioned among the presently identified lipoprotein fractions in a manner that may be powerfully revealing.[12-15,27,29,33,34,41]

6. Are there tissues that accumulate oxysterols in a manner suggesting association with function, dysfunction, or disease?

The presence of oxysterols in mammalian tissues has been demonstrated, and in several instances accumulations of oxysterols have been discovered. The accumulation of the 3β,26-diol *11* and of fatty acyl esters of 24-, 25-, and 26-hydroxycholesterols in the human aorta with increasing age and severity of associated atherosclerosis[42] and the increase in (24S)-cholest-5-ene-3β,24-diol levels of rat brain following myelinization[43] suggest these oxysterols have some relationship to the status of these tissues in vivo. Increased amounts of oxysterol fatty acyl esters in tissues from Tangier disease patients[44] may or may not have significance, as the analyzed tissues had been frozen for years before analysis, and no measure of autoxidation occurring during storage was had.

The amount of the 5β,6β-epoxide *8* in human breast fluid appears to fluctuate with hormonal balance of women donors without breast cancer.[45]

7. Are the established in vitro toxic effects indeed expressed in vivo?

Several in vitro effects of oxysterols have been demonstrated in vivo as well. Whether in vivo toxic effects are the same or similar to in vitro effects has yet to be determined, although some effects such as in vitro inhibition of HMGCoA reductase occur also in vivo.

8. Are oxysterols physiologically relevant in essential or beneficial metabolic processes or do they pose a genuine threat to human health?

Guidance of this issue may be provided, but the question cannot now be answered. Disputation either way can be found, but uncertainties abound. Whether the oxysterols toxicities be implicated in the etiologies of chronic human diseases (atherosclerosis, cancer), whether, provocatively, toxic tissue and plasma oxysterols provide natural protection against invading viruses, microorganisms, or malignant tumor cells, or whether oxysterols have no physiological significance remain uncertainties yet to be resolved.

An hypothesis formulated by Chen and Kandutsch over a decade ago that certain oxysterols serve as endogenous regulators of de novo sterol biosynthesis via their inhibitory effects of the regulatory enzyme HMGCoA reductase continues to attract adherants. It may be that this biological action be one of the few beneficial actions of oxysterols.

Obviously the oxysterol metabolites of cholesterol implicated in established biosynthesis processes for the cholestanols (3-ketone *9*), bile acids (3β,7α-diol *2*, 3β,26-diol *11*), and steroid hormones (3β,20-diol *10*, (22R)-cholest-5-ene-3β,22-diol) whether also formed by adventitious oxidation processes or not fall outside the present issue of benevolence, being required for mammalian life.

Although there are new in vivo data here reviewed, much recent work with oxysterols has been with cultured mammalian cells under conditions where in vivo implications remain uncertain. Another limitation of in vitro bioassay of oxysterols lies in the commercially available samples actually used. The notorious state of purity of commercial samples for reliable bioassay purposes is often ignored, and bioassay results on such samples may be compromised accordingly.

Bioassay of the 5α,6α-epoxide *5* in most cases involves use of commercially available material known to contain as much as 10–15% 5β,6β-epoxide *8*. Whereas mutagenicity bioassays of these samples most likely do measure the mutagenicity of the predominant 5α,6α-epoxide *5*, it now appears that the 5β,6β-epox-

ide *8* is also mutagenic,[46,47] so the full measure of biological activity of either epoxide may be uncertain. Where pure samples of both 5,6-epoxides have been prepared for bioassay, data establish that the individual 5,6-epoxides *5* and *8* are both cytotoxic and that both possess transforming ability on cultured mammalian cells.[48]

Some of the prior conflicting and confusing tests of the 5α,6α-epoxide *5* may have come from use of impure samples. For instance, the inhibition of DNA synthesis by impure 5α,6α-epoxide *5* in cultured Syrian hamster embryo cells could not be demonstrated with pure samples, whether with or without the liver microsomal S-9 enzymes activation system.[49,50] However, inhibition of DNA synthesis in Chinese hamster V79 lung fibroblasts by either 5,6-epoxide *5* or *8* has since been demonstrated.[46,47] Furthermore, we have experienced the same wasted effort by testing commercial samples of cholest-4-ene-3,6-dione and 3β,5-dihydroxy-5α-cholestan-6-one for mutagenicity against *Salmonella typhimurium*.[51] The efforts necessary later to confirm spurious activities found with impure samples far exceeds the effort required to prepare pure samples initially. Careful workers elsewhere have had related experiences.[52,53]

Obviously aged samples of stigmasterol containing progesterone as an autoxidation product[54] might be bioassayed as hormonally active! Nonetheless, the purity of samples tested is not revealed in most cases, and identities are almost always by suppliers labels! Bioassay results are also influenced by the means by which oxysterols are presented. In vitro bioassays employing serum or serum lipoproteins are notoriously influenced by such media.[41,55-58]

Another major limitation for either in vitro bioassay or in vivo testing is that of metabolism of the analyte during bioassay. This limitation is most obvious for sterol hydroperoxides and peroxides, where protective metabolism reducing or destroying the peroxide bond may occur. Thus, cholesterol 7α-hydroperoxide and 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide are metabolized by *Salmonella typhimurium* TA1537 employed in bioassay for mutagenicity.[59,60] The Vitamin D_3 peroxides active in inducing differentiation of human myeloid leukemia HL-60 cells also are metabolized by the cultured cells, in this case to the active agents.[61,62] The immunosuppresion activity of cholesterol 25-hydroperoxide against cultured mouse spleen cells might also be attributed to its reduction product 3β,25-diol *7* equally active in the test.[63]

In other cases genuine biological activity of steroid hydroperoxides (and not of the corresponding reduced derivative) appears the case. For instance, cholesterol 25-hydroperoxide but not the 3β,25-diol *7* appears to inhibit the action of calmodulin.[64] Also, kinetics data showing that the human placental aromatase (cytochrome $P450_{AROM}$) inhibitor 10β-hydroperoxyestr-4-ene-3,17-dione binds five-fold greater than the corresponding 10β-hydroxyestr-4-ene-3,17-dione suggests that the 10β-hydroperoxide be the reactive agent oxidizing the active site sulfhydryl group of the enzyme.[65] Other steroid hydroperoxides 6α- and 6β-hydroperoxyandrost-4-ene-3,17-diones[66] and 17α-ethinyl-10β-hydroperoxy-17β-hydroxyestr-4-en-3-one[67] also are active aromatase inhibitors in the same fashion. Moreover, 10β-hydroperoxyestra-1,4-diene-3,17-dione is implicated as the active agent in the photosensitized binding of estrone (3-hydroxyestra-1,3,5(10)-trien-17-one) to protein or to DNA.[68,69]

Yet other similar concerns for the metabolism of nonperoxide oxysterols during bioassay have been expressed, the isomeric 5,6-epoxides *5* and *8* both being subject to metabolic hydrations to the 3β,5α,5β-triol *6* during bioassay.[46,47] As the 5β,6β-epoxide *8* appears to be more mutagenic than 5α,6α-epoxide *5* and 3β,5α,6β-triol *6* is nonmutagenic but more toxic than either 5,6-epoxide, bioassay results are the sum of the several metabolic and toxicity effects taking place.

It appears that metabolism of the HMGCoA reductase inhibitor 3β-hydroxy-5α-cholest-8(14)-en-15-one (*12*) by cultured Chinese hamster ovary CHO-K1 cells used for bioassay does not interfere with the bioassay despite transformation of the 15-ketone *12* to cholesterol by other systems.[70] However, in other cases cultured mammalian cells do metabolize exogenous oxysterols to biologically active products![71,72]

In these cases one may see metabolic activation, deactivation, or both, and the true potency of any given oxysterol in any bioassay thus is uncertain, as so few reports of metabolism during bioassay have been made. In most bioassay studies with cultured cells where analysis of the system during or after bioassay for oxysterols is so easy, little attention has been given to this limitation. Necessarily, the issue of in vivo physiological relevance of oxysterols depends on any metabolic disposition the oxysterol may experience. These matters are undertended at present.

The literature reviewed here on balance confirm and reinforce viewpoints summarized in 1981 that oxysterols as a class be toxic agents. Nonetheless, four salient thrusts that have developed from issues previously set forth that bear on this issue deserve special mention at this point:

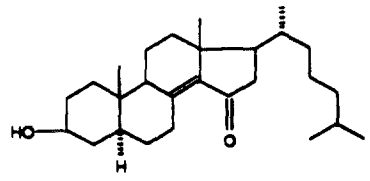
1. Cholesterol hydroperoxide and epoxide derivatives have been demonstrated mutagenic in vitro, and

the isomeric 5,6-epoxides have been shown to possess transforming activity against cultured cells.

2. A single though impressive report that oxysterols be no more atherogenic than cholesterol in vivo has initiated controversy on this point.

3. Oxysterols such as the 3β,7β-diol *3* 3β,7β-dihemisuccinate provide in vivo protection against malignant tumors, and the 15-ketone *12* proposed as chemotherapeutic agents for lowering plasma cholesterol levels by inhibition of de novo sterol biosynthesis is effective in vivo.

4. An hypothesis that endogenous intracellular oxysterols such as the 3β,25-diol *7* or 24,25-epoxy-(24S)-cholest-5-en-3β-ol (desmosterol 24,25-epoxide, *13*) be endogenous regulators of de novo sterol biosynthesis has gained additional experimental attention.



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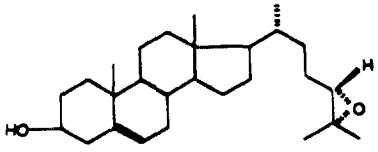

13 Desmosterol 24, 25 - epoxide

These four developments and our recent demonstration that active oxygen species be implicated in the expression of toxic effects of sterol hydroperoxides add dimension to the importance of continued study of the biochemistry and biological activities of oxysterols for eventual discovery of their true physiological relevance to human health.

CYTOTOXICITY

The in vivo and in vitro toxicities of oxysterols reviewed previously[1] are on balance their predominant biological characteristic. The present section examines generalized toxic effects in vivo and effects on survival, growth, cell proliferation, and function in vitro. Review of the more specific in vivo toxic manifestations of atherogenicity, mutagenicity, and carcinogenicity and of effects on cellular membranes and specific enzyme system is treated in succeeding sections.

In vivo toxic effects may result from diverse and obscure reasons, not necessarily being specifically caused by administered oxysterols. Thus, where the oxysterol serves as sole sterol source in the diet (observed for ergosterol peroxide (5,8-epidioxy-5α,8α-(22E)-ergosta-6,22-dien-3β-ol), 3β,7β-diol *3*, 7-ketone *4*, 3β,20-diol *10*, 3β,25-diol *7*, and 15-ketone *12*), the killing of silkworm *Bombyx mori* larvae may not be so much a toxic effect as starvation resulting from failure of the larvae to metabolize oxysterol. When fed 0.01% oxysterol with 0.1% cholesterol survival and growth were not significantly affected.[73]

Moreover, although this review of cytotoxicity deals directly with specific oxysterols preparations, it must be recognized that the process of peroxidation of sterols involving oxyl, peroxyl, and carbon-centered radicals as well as stable oxysterol products may also contribute to toxic effects. For example, cholest-5-en-3-one, Vitamin D_3 and D_2 (ergocalciferol, (5Z,7E,22E)-9,10-secoergosta-5,7,22-trien-3β-ol), ergosterol ((22E)-ergosta-5,7,22-trien-3β-ol), and cholesta-5,7-dien-3β-ol, all more readily autoxidized than cholesterol, exert a prooxidative effect on polyunsaturated fatty acyl esters, whereas cholesterol produced no such prooxidative effect.[74] Indeed, cholesterol may be protective against peroxidant stresses in vivo.[75,76]

In vivo toxicities

Toxicities are manifested at the cellular level in diminished cellular functions, growth, and proliferation and in vivo in cessation of growth, loss of weight, and diminished appetite but also in pathological changes, including damage to arteries and aortal lesions of atherosclerosis. Table 1 summarizes accounts of in vivo toxicities of dietary oxysterols.

Toxic effects are also elicited via oxysterols administration by other means. Injection of the 7-ketone *4* in rabbits resulted in lowering of plasma cholesterol levels;[98] subcutaneous implantations of the 3β,5α,6β-triol *6*, 3β,25-diol *7*, or 3β,26-diol *11* in rats led to necrosis and an acute inflammatory response;[99,100] rectal instillation of 3-ketone *9* or 5α-cholestan-3-one in mice generated nuclear aberrations in the colon mucosa.[101] Ergosterol peroxide inuncted onto chick embryos is toxic, and when added to water is toxic to brine shrimp.[102] Plasma lipoproteins treated with cholesterol oxidase becomes lethal upon injection into rabbits, but it is uncertain whether the effect be attributed to the 3-ketone *9* formed by enzyme action.[103]

Oxysterols have proven toxic to microorganisms. Cholesterol 7α-hydroperoxide, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide, and the 3β,25-diol *7* are bacteriacidal to *Salmonella typhimurium*[51,59] and the 3β,5α,6β-triol *6* and 3β-hydroxy-5α-cholestan-6-one inhibit growth of *Mycoplasma gallisepticum*.[104] Ad-

Table 1. In Vivo Effects of Dietary Oxysterols

Oxysterol	In Vivo Effects	Reference
Cholesterol 7α-hydroperoxide, 0.2% in diet	Supports induction of cytochrome P450 enzymes in male albino rats (but no more than cholesterol)	77
Cholest-5-ene-3β-,7α-diol *2* 3,7-dihemisuccinate	Toxic in Swiss mice, LD_{50} 0.3 mmole/kg	78
Cholest-5-ene-3β,7β-diol *3* 3,7-dihemisuccinate	Toxic in mice, LD_{50} 0.17 mmole/kg	79
3β-Hydroxycholest-5-en-7-one *4*, 0.025% in diet	Decreased HMGCoA reductase in laying hens	80–81
5,6α-Epoxy-5α-cholestan-3β-ol *5*	Increased bile acids excretion; no effect on *de novo* sterol biosynthesis	28
5α-Cholestane-3β,5,6β-triol *6*, 1% in diet	Loss of appetite in $B6C3F_1$ mice	82
5β-Cholestane-3α,5,6α-triol*	Depressed food intake in gerbils but not in chicks	83, 84
Cholest-4-en-3-one *9*, 1% in diet 3.5 months	Retarded growth, enlarged adrenals, in male Wistar rats; growth retardation and hyperplastic ovaries in female Wistar rats; other effects	85
Cholest-4-en-3-one 9, 1% in diet	Lipoid adrenal hyperplasia in male Wistar rats Male Sprague–Dawley rats, adrenal hypertrophy, depressed corticosterone production	86 87
5,6β-Epoxy-5β-cholest-7-en-3β-ol	Toxic in ICB mice, ID_{50} ca. 140 mg/kg	88
Commercial cholesterol, containing oxysterols 200 mg/kg 6 weeks	Increased blood cholesterol levels in Chinchilla rabbits	89
Mixed oxysterols, 0.5% in diet	Decreased HMGCoA reductase in laying hens; no effect on body weight or egg production and weight; reduced acetate incorporation into yolk sterol	80, 90, 91
Mixed oxysterols, 0.5% in diet 13–14 weeks	Lethal to chicks (6/16) and to young quail (5/17)	92
Mixed oxysterols, 1% in diet 3 weeks	Reduced feed consumption, weight, and serum cholesterol in female quail; reduced serum cholesterol in male quail	93
(17(20)Z)-Cholesta-5,17(20)-dien-3β-ol, 0.25% in diet 26 h	Decreased appetite and diminished *de novo* sterol biosynthesis in male C57BL/6J mice	94
3β-Hydroxy-5α-cholest-8(14)-en-15-one (*12*)	Loss of appetite, weight loss in rats	95
3β-Hydroxy-5α-lanost-8-en-7-one, 0.1% in diet	Lowered serum cholesterol and triacylglycerols in male Wistar rats	96
3β-Benzoyloxy-5α-stigmastane-5,6β-diol, 680 mg/kg orally	Minor reduction in hemoglobin, simple chromosomal aberrations in Swiss albino mice	97

*The oxysterol implicated is almost certainly the 3β,5α,6β-triol *6*.

ditionally, 21-nor-(20ξ)-cholest-5-ene-3β,20-diol and 3β-hydroxy-21-norcholest-20-one inhibit the Δ^{22}-dehydrogenase of *Tetrahymena pyriformis*[105] and (25ξ)-26-norcholest-5-ene-3β,25-diol and 3β-hydroxy-26-norcholest-5-en-25-one inhibit growth of the *Saccharomyces cerevisiae* auxotroph RD5-R.[106]

In vitro toxicities

By far the most obvious effort has been placed on studies of the effects of oxysterols on cultured mammalian cells (Table 2). Whether these in vitro effects have any relationship to physiological reality generally remains untested. Moreover, in cases where several related cell lines have been examined under different culture (medium) conditions different sensitivities of cells to oxysterols have been observed,[115] so extrapolation of specific in vitro results to other situations is uncertain.

These manifestations of toxicity, though diverse, may have at least two separate origins. The long recognized inhibition of HMGCoA reductase activity accounts for those toxicities in which cell requirements for sterol are not met, but the incorporation of oxysterol into plasma membranes affecting membrane fluidity, function, and stability is emerging as a second major mode of provoking toxicity.[104,118,127]

Table 2. In Vitro Cytotoxic Effects of Oxysterols

Oxysterol	In Vitro Results	Reference
5,6α-Epoxy-5α-cholestan-3β-ol *5*	Chinest hamster embryo cells, plating efficiency decreased*	107, 108
	Chinese hamster V79 lung fibroblasts, cytotoxic at 20–25 μM	47, 48
	Syrian hamster embryo cells, toxic, TD_{50} 10 μg/mL	109
	Rat Morris hepatoma HTC cells, moderate inhibition of growth	110
	C3H-10T1/2 mouse embryo cells, toxic, at 20 μM	48
	Mouse L cells, cytotoxic, reduced protein levels	111
5,6β-Epoxy-5β-cholestan-3β-ol *8*	Chinese hamster V79 lung fibroblasts, toxic at 20 μM	47, 48
	Rat Morris hepatoma HTC cells, moderate inhibition of growth	110
	C3H-10T1/2 mouse embryo cells, toxic at 20 μM	48
	Mouse L cells, cytotoxic, lipid droplet accumulations	111
3β-Hydroxy-24-ethyl-(24ξ)-cholesta-5,28-diene-24-hydroperoxide	Murine leukemia L1210 cells, toxic, ID_{50} 2 μg/mL	112
3β-Hydroxy-24-ethylcholesta-5,23-diene-28-hydroperoxide	Murine leukemia L1210 cells, toxic, ID_{50} 4 μg/mL	112
3β-Hydroxy-24-ethylcholesta-5,24(28)-diene-29-hydroperoxide	Murine leukemia L1210 cells, toxic, ID_{50} 4 μg/mL	112
5,8-Epidioxy-5α,8α-(22E)-ergosta-6,22-dien-3β-ol (ergosterol peroxide)	Mouse lymphemia L-1210/v/c cells, toxic, IC_{50} 3.5 μg/mL	113
5,8-Epidioxy-5α,8α-cholest-6-en-3β-ol	Mouse lymphemia L-1210/v/c cells, toxic, LD_{50} 11.7 μg/mL	114
	Human epidermoid carcinoma KB cells, toxic, LD_{50} 12.3 μg/mL	114
5,8-Epidioxy-6β,7β-epoxy-5α,8α-cholestan-3β-ol	Mouse lymphemia L-1210/v/c cells, toxic, LD_{50} 3.5 μg/mL	114
5α-Cholestane-3β,6β-diol	Rat Morris hepatoma HTC cells, moderate inhibition of growth	110
Cholest-5-ene-3β,7α-diol *2*	Chinese hamster embryo cells, relative plating efficiency decreased	108
	Rat Morris hepatoma HTC cells, moderate inhibition of growth	110
	Mouse L cells, cytotoxic, reduced protein levels, lipid droplet accumulations	111
Cholest-5-ene-3β,7β-diol *3*	Chinese hamster embryo cells, relative plating efficiency decreased	108
	Rat Morris hepatoma HTC cells, lysis by 24 h at 20 μM, lysis by 72 h at 10 μM	57
	Murine lymphoma EL-4, YAC-1, RDM4 cells, toxic at 3-12 μM	115
	Rat fibroblasts, toxicity	72, 116
	Rat myocardial cells, synchronous beating impaired by 2.5 μM	72, 116
	Mouse L cells, cytotoxic, reduced protein levels, lipid droplet accumulations	111
(20S)-Cholest-5-3β,20-diol *10*	Bovine platelets, thrombin-induced aggregation increased 151% by 25 μM	117
	Human marrow mononuclear cells, proliferation inhibited, ID_{50} <0.50 μM	118
	Bovine platelets, thrombin-induced aggregation increased 162% by 25 μM	117
(22R)-Cholest-5-3β,22-diol	Bovine platelets, thrombin-induced aggregation increased 189% by 25 μM	117
	Rat Morris hepatoma HTC cells, lysis by 48 h at 40 μM; lysis by 72 h at 20 μM	57
(24R)-Cholest-5-ene-3β,24-diol	Bovine platelets, thrombin-induced aggregation increased 182% by 25 μM	117
(24S)-Cholest-5-ene-3β,24-diol	Bovine platelets, thrombin-induced aggregation increased 229% by 25 μM	117
	Rat Morris hepatoma HTC cells, moderate inhibition of growth	110
Cholest-5-ene-3β,25-diol *7*	Human skin fibroblasts, sterol biosynthesis inhibited 95% by 1.25 μM; cytotoxic at 20 μg/mL	119, 120
	Human artery smooth muscle cells, cytotoxic at 20 μg/mL	120
	Preconfluent keratinocytes, sterol biosynthesis inhibited 84% by 1.25 μM	119
	Human marrow mononuclear cells, toxic ID_{50} 0.54 μM	118
	Bovine platelets, thrombin-induced aggregation increased 165% by 25 μM	117
	Pig vascular smooth muscle cells, toxic	100
	Rat prostate adenocarcinoma P-III, colony inhibition 75.7% at 6 μg/mL†	121
	Rat C-6 glioma cells, toxic at 2.5 μM	122
	Mouse fibroblasts, toxic	100
	Mouse peritoneal macrophages, toxic	100
	Mouse L cells, cytotoxic, reduced protein levels, lipid droplet accumulations	111
	Mouse lymphocytes, cytotoxic 1 μg/mL	123
3β-Hydroxy-5α-cholestan-6-one	Human marrow mononuclear cells, proliferation inhibited, ID_{50} 5.0 μM	118
	Mouse L cells, cytotoxic, reduced protein levels, lipid droplet accumulations	111
3β-Hydroxycholest-5-en-7-one *4*	Rat Morris hepatoma HTC cells, toxic within 24 h	110
	Human marrow mononuclear cells, proliferation inhibited, ID_{50} 5.0 μM	118
	Mouse L cells, cytotoxic, reduced protein levels, lipid droplet accumulations	111
	Murine neuroblastoma cells, sterol biosynthesis and HMGCoA reductase markedly inhibited at 5 μg/mL	124
	Chinese hamster V79 lung fibroblasts, cytotoxic at 25 μM	47, 125
Cholesta-3,5-dien-7-one	Mouse L cells, cytotoxic, reduced protein levels, lipid droplet accumulations	111
3β-Hydroxy-5α-cholestan-7-one	Chinese hamster V79 lung fibroblasts, cytotoxic at 10 μM	47
3β-Hydroxycholest-5-en-22-one	Human marrow mononuclear cells, proliferation inhibited, ID_{50} 25 μM	118

Table 2. (Continued)

Oxysterol	In Vitro Results	Reference
3β-Hydroxycholest-5-en-24-one	Rat Morris hepatoma HTC cells, toxic within 1–3 d	110
3β-Hydroxy-24-ethylcholesta-5,24(28)-dien-29-al	Murine leukemia L1210 cells, toxic, LD_{50} 3 μg/mL	112
24-Ethylcholesta-5,24(28)-diene-3β,29-diol	Murine leukemia L1210 cells, toxic, ID_{50} 4 μg/mL	112
3β,5-Dihydroxy-5α-cholestan-6-one	Rat Morris hepatoma HTC cells, toxic at 33 μg/mL	126
3β,5-Dihydroxy-5α-cholest-7-en-6-one	Rat Morris hepatoma HTC cells, toxic at 33 μg/mL	126
5α-Cholestane-3β,5,6β-triol *6*	Bovine platelets, thrombin-induced aggregation decreased by 25 μM	117
	Pig vascular smooth muscle cells, toxic	100
	Chinese hamster embryo cells, relative plating efficiency decreased*	107, 108
	Chinese hamster V79 lung fibroblasts, cytotoxic at 12 μM	47
	Syrian hamster embryo cells, toxic, TD_{50} 2.3 μg/mL	109
	Mouse fibroblasts, toxic	100
	Mouse peritoneal macrophages, toxic	100
6β-Methoxy-5α-cholest-7-ene-3β,5,9α-triol	Rat Morris hepatoma HTC cells, toxic at 33 μg/mL	126
3β,5,9α-Trihydroxy-5α-cholest-7-en-6-one	Rat Morris hepatoma HTC cells, toxic at 33 μg/mL	126
Mixed oxysterols	Chinese hamster embryo cells, relative plating efficiency decreased†	107, 108

*Effect mitigated by exogenous antioxidants.
†Effect mitigated by exogenous cholesterol.

A serious limitation of many in vitro studies is the inclusion of serum, serum lipids, or serum lipoproteins in media used. The presence of serum components influences cell growth, proliferation, and function in major ways but appears in general to moderate the effects of exogenous oxysterols. At the same time, serum preparations commonly used may contain unrecognized amounts of oxysterols that may influence results in some cases.[128,129] The controlling influences of lipoprotein on in vitro expressions of oxysterols toxicity may come at least in part from the partitioning of oxysterols among the different lipoprotein fractions, which fractions diminish insertion of oxysterols into plasma membranes and subsequent oxysterol toxicity.[41,55] Thus, as one must consider the circumstances under which cholesterol is autoxidized in biological systems,[1,130] so also the conditions of bioassay of oxysterols for toxicity are of importance.

Despite these problems the indicated in vitro toxicities suggest useful medical applications, including exploitation of oxysterol cytotoxicity for control of tumor cell growth, proliferation, and survival and of oxysterol suppression of de novo sterol biosynthesis as means of reducing plasma cholesterol levels in vivo. As example of potential usefulness as an antitumor agent, the 3β,7β-diol *3* and its 3β,7β-dihemisuccinate sodium salt are toxic to cultured undifferentiated rat Morris hepatoma HTC cells, but both are relatively inactivated by serum lipoproteins.[58,78,115] Nonetheless, the 3β,7β-diol *3* 3β,7β-dihemisuccinate provided protection against Krebs II tumors in mice faster and more strongly than other antitumor agents such as cyclophosphoramide, 5-fluorouracil, or methotrexate![58,78]

The 3β,7β-diol *3* is uniformly toxic to cultured murine lymphoma cells but is not toxic to normal mouse lymphocytes. Indeed, the 3β,7β-diol *3* may exert a protective effect on lymphocytes at high (40–50 μM) concentrations.[115] Were such 3β,7β-diol *3* levels chronically protective in vivo, one may pose speculatively the general case, that plasma oxysterols be protective against cancer cells survival within the circulation, thus providing speculatively a basis for the presence of the 3β,7β-diol *3* and other oxysterols in human blood. Human plasma 3β,7β-diol *3* levels of 0.18–4.1 (average 1.7) μM have been reported,[39] and 3β,7β-diol *3* fatty acyl esters occur in human blood at 11 ng/mL (ca. 15 nM), thus not much lower than the 3–12 μM levels toxic to cultured lymphomas in vitro. Moreover, plasma levels of the 3β,7α-diol *2* have been measured at comparable (0.3–2.5 μM) levels,[39] and plasma 3β,26-diol *11* levels have been reported in the range 0.1–2.3 μM.[15,36–39] It is not known whether these plasma oxysterols are also cytotoxic to cancer cells or protective of lymphocytes. Moreover, as the lymphoma toxicity data depended on medium composition, toxicity in vivo cannot be projected.

Programs for synthesis of new cytotoxic oxysterols as potential antitumor agents (using Morris hepatoma HTC cells for bioassay) and for improved syntheses of established ones have been conducted.[131–133] Such synthetic oxysterol analogs as 6-nitrocholest-5-en-3β-ol, 22-oximinocholest-5-en-3β-ol, 3β-hydroxy-5α-cho-

lest-8-en-7-one, 3β-hydroxy-5α-cholest-8-en-11-one, 3β-hydroxy-5α-cholest-8(14)-en-7-one, 3β-hydroxy-4,4-dimethylcholest-5-en-3β-ol, 4,4-dimethylcholest-5-ene-3β,7α-diol and 4,4-dimethylcholest-5-ene-3β,7β-diol are all inhibitory.[134]

Other in vitro activities

Although several biological activities of oxysterols may not be readily classified as toxicities, it is convenient to include these cases in this section as all are in vitro studies involving cultured cells and cell survival, growth, and proliferation. One such important activity is the suppression of DNA biosynthesis as measured by thymidine incorporation (Table 3). Oxysterol inhibition of DNA synthesis in vitro is accompanied by suppression of de novo sterol biosynthesis, and DNA synthesis appears dependent upon the availability of sterol, as exogenous sterol, lipoprotein, or serum relieve the effect. However, suppression of DNA and sterol biosyntheses may be separated in some cases.[149] The lack of sterol is associated with arrest of cells at

Table 3. Oxysterols Inhibitions of DNA Biosynthesis

Oxysterol	In Vitro System	Reference
Cholest-5-ene-3β,7α-diol *2*	Human lymphocytes, 1–5 μg/mL	135, 136
Cholest-5-ene-3β,7β-diol *3*	Human lymphocytes, 5 μg/mL	135
	Rat Morris hepatoma HTC cells	56
	Rat embryo fibroblasts, 23% inhibition by 6 μM	137
	Mouse splenocytes, 1–5 μg/mL	138
Cholest-5-ene-3β,7β-diol *3* 3β,7β-dihemisuccinate	Rat Morris hepatoma HTC cells	56
3β-Hydroxycholest-5-en-7-one *4*	Human peripheral blood mononuclear cells, lymphocytes	135, 139
	Rat embryo fibroblasts, 13% inhibition by 6 μM	137
	Mouse lymphocytes, 0.5–2 μg/mL	140
(22R)-Cholest-5-ene-3β,22-diol	Human lymphocytes, 24% at 25 μg/mL	141
(22S)-Cholest-5-ene-3β,22-diol	Human lymphocytes, 7% at 25 μg/mL	141
(23R)-Cholest-5-ene-3β,23-diol	Human lymphocytes, 17% at 25 μg/mL	141
	Rat embryo fibroblasts, 8% by 6 μM	137
(23S)-Cholest-5-ene-3β,23-diol	Human lymphocytes, 79% at 25 μg/mL	141
	Rat embryo fibroblasts, 50% by 6 μM	137
(24R)-Cholest-5-ene-3β,24-diol	Human lymphocytes, 49% at 25 μg/mL	141
(24S)-Cholest-5-ene-3β,24-diol	Human lymphocytes, 93% at 25 μg/mL	141
Cholest-5-ene-3β,25-diol *7*	Human lymphocytes, 90% at 25 μg/mL; 14% at 5 μg/mL	139, 141–144
	Human WI-38 fibroblasts	145
	Human smooth muscle cells, inhibited at 2 μg/mL	146
	No effect in monkey artery smooth muscle cells	147, 148
	Rat embryo fibroblasts, 92% inhibition by 6 μM	137, 149
	Rat L_6 myoblasts	145
	Mouse spleen cells, by 2 μg/mL	123
	Murine P815 mastocytoma cells, 0.1–1 μg/mL	150
(25R)-Cholest-5-3β,26-diol *11*	Human lymphocytes, 85% at 25 μg/mL	141
	Rat embryo fibroblasts, 63% by 6 μM	137
Cholesta-5,23-diene-3β,25-diol	Human lymphocytes, 97% at 25 μg/mL; 28% at 5 μg/mL	141
Cholesta-5,20(22)-diene-3β,25-diol	Rat embryo fibroblasts, 48% inhibition by 6 μM	137
Cholest-5-ene-3β,7β,25-triol	Mouse splenocytes	151
(5Z,7E)-9,10-Secocholesta-5,7-diene-3β,25-diol (25-hydroxy-Vitamin D_3)	Human lymphocytes, 65% at 25 μg/mL; 14% at 5 μg/mL	141
3β-Hydroxy-24-ethyl-(24ξ)-cholesta-5,28-diene-24-hydroperoxide	DBA/2 mouse lymphocytes, ID_{50} 1 μg/mL	112
3β-Hydroxy-24-ethylcholesta-5,23-diene-28-hydroperoxide	DBA/2 mouse lymphocytes, ID_{50} 4 μg/mL	112
3β-Hydroxy-24-ethylcholesta-5,24(28)-diene-29-hydroperoxide	DBA/2 mouse lymphocytes, ID_{50} 16 μg/mL	112
3β-Hydroxy-24-ethylcholesta-5,24(28)-dien-29-al	DBA/2 mouse lymphocytes, ID_{50} 1 μg/mL	112
24-Ethylcholesta-5,24(28)-diene-3β,29-diol	DBA/2 mouse lymphocytes, ID_{50} 4 μg/mL	112
(5Z,7E,24R)-9,10-Secocholesta-5,7-diene-3β,24,25-triol	Human lymphocytes, 73% at 25 μg/mL; 14% at 5 μg/mL	141
(5Z,7E)-9,10-Secocholesta-5,7-diene-1α,3β,25-triol (1α,25-dihydroxy-Vitamin D_3)	Human lymphocytes, 52% at 25 μg/mL, 19% at 5 μg/mL	141

the G_1 phase of the cell cycle, and its appears that the G_1 arrest preceed, indeed lead to suppression of subsequent macromolecule biosyntheses.[150]

Yet other data implicate membrane phenomena in suppression of DNA synthesis. Thus, the 3β,7β-diol *3* and 3β,25-diol *7* but not the water soluble 3β,7β-diol *3* 3β,7β-dihemisuccinate suppress DNA synthesis in murine splenocytes. Incorporation of oxysterol into the plasma membrane may contribute to the effect.[139] Also, the increased uptake of Ca^{2+} and decreased DNA synthesis in murine P815 mastocytoma cells treated with 0.1–1 μg/mL 3β,25-diol *7* suggest that DNA synthesis may be inhibited as Ca^{2+} influx be stimulated via membrane events.[151]

There are divergent responses for some epimeric hydroxycholesterols; for instance, while the epimeric cholest-5-ene-3β,24-diols are equally inhibitory of DNA synthesis at lower dose, the naturally occuring (24S)-cholest-5-ene-3β,24-diol is almost twice as inhibitory at higher dose.

Oxysterols exert other actions in suppressing cell proliferation, including an in vivo antimitotic activity. Intravaginal instillation of the 7-ketone *4* or 3β,25-diol *7* in mice during the progesterone-dominant phase of the estrus cycle prevented metaphase figure formation in vaginal epithelium, but the effect was local as rectal epithelium was not affected.[152]

Oxysterols are also potent immunosuppressive agents affecting both generation of cytotoxic lymphocytes and their function, by inhibiting lymphocyte proliferation and transformation (blastogenesis), the mixed lymphocyte response, and the activity of natural killer cells. Cholesterol 25-hydroperoxide and the 3β,25-diol *7* are immunosuppressive agents.[63] The 3β,25-diol *7* suppresses concanavalin A-, mevalonate-, and phytohemagglutinin-induced transformations of human lymphocytes[143,153] and inhibits the transformation of naive murine lymphocytes into differentiated cytotoxic lymphocytes.[123] The 3β,7-diols *2* and *3* and 7-ketone *4* were likewise effective in phytohemagglutinin-stimulated human peripheral blood lymphocytes.[135,136,153] Indeed, 3β,7-diols *2* and *3* administered intraperitoneally (10–50 mg/kg/day) to rats with skin grafts appeared to prolong graft survival times.[135]

The epimeric 3β,7-diols *2* and *3* are both potent suppressors of cytotoxic lymphocyte responses,[154] and the doubly substituted oxysterol cholest-5-ene-3β,7,25-triol was also immunosuppressive, inhibiting blastogenesis and mixed lymphocyte response but only about as much as the 3β,7β-diol *3*.[151] The 7-ketone *4* (25 μM) or 3β-hydroxy-5α-cholestan-7-one also inhibited spleen cell cytotoxicity and natural killer cell activity.[155]

These suppressor actions appear to derive from inhibition of de novo sterol biosynthesis, as cholesterol is required for proliferation and cytotoxicity of these cells.[123,136,139,142,145] The effects of the 7-ketone *4* in inhibiting lymphocyte blastogenesis appear to depend on the cholesterol:phospholipid ratio in the plasma membrane.[140] Nonetheless, as in so many other cases of oxysterols influences, more than one mechanism may operate. For instance, the 3β,7β-diol *3* suppresses murine splenocyte secretion of interleukin-2 and the appearance of interleukin-2 receptors in human T lymphocytes, both matters being required for final cell proliferation.[138] Moreover, the 3β,25-diol *7* stimulates biosynthesis of apolipoprotein E in murine peritoneal macrophages, apolipoprotein E also being recognized as inhibiting lymphocyte proliferation.[156]

In that the irradiation of skin in vivo leads to diminished immune system responses and also may generate oxysterols in situ, it is speculated that compromised immune system responses may result from immunosuppressive oxysterols so formed in irradiated skin.[154]

Among other diverse activities of oxysterols is the induction of differentiation of human myeloid leukemia HL-60 cells, the differentiation actually being measured as an increase in phagocytosis by the HL-60 cells. Here several oxidized derivatives of 25-hydroxy-Vitamin D_3 ((5Z,7E)-9,10-secocholesta-5,7,10(19)-triene-3β,25-diol) have pronounced activities (cf. Table 4). However, the induction activity was not correlated with binding of oxysterols to cytosol receptor for 1α,25-dihydroxy Vitamin D_3 ((5Z,7E)-9,10-secocholesta-5,7-diene-1α,3β,25-triol).[61]

Oxysterols are also rapidly effective in influencing thrombin- or adenosine diphosphate-induced aggregation of bovine blood platelets in vitro. Oxysterols (25 μM) bearing a side-chain hydroxyl feature ((20R)-cholest-5-en-3β-ol, 3β,20-diol *10*, the isomeric cholest-5-ene-3β,22-diols and cholest-5-ene-3β,24-diols, and 3β,25-diol *7*) enhanced thrombin-induced aggregation, but only (22S)-cholest-5-ene-3β,22-diol enhanced adenosine diphosphate-induced aggregation, whereas (22R)-cholest-5-ene-3β,22-diol, (24S)-cholest-5-ene-3β,24-diol, and the 3β,25-diol *7* inhibited aggregation. Other common cholesterol autoxidation products 7-ketone *4* and 5α,6α-epoxide *5* were not active, but the 3β,5α,6β-triol *6*, and 3β,5-dihydroxy-5α-cholestan-6-one inhibited thrombin-induced aggregations.[117,157,158] The means by which these actions occur remain uncertain.

However, the related phenomenon of attachment of cultured Chinese hamster lung Dede cells, Chinese hamster ovary cells, and mouse L cells to serum-coated glass prevented in a dose-dependent fashion by 3β,7β-diol *3*, 3β,20-diol *10*, 3β,25-diol *7*, 3β-hydroxycho-

Table 4. Phagocytosis Activities of Oxysterols

Oxysterol	Increased Phagocytosis, %*	Relative Potency†
6,19-Epidioxy-(7E)-9,10-secocholesta-5(10),7-diene-1α,3β,25-triols	ca.15-25 (ca.100 nM) ca.55-60 (ca.600 nM)	130-200
6,19-Epidioxy-26,27-hexafluoro-(7E)-9,10-secocholesta-5(10),7-diene-3β,25-diols	ca.15 (10 nM) ca.55 (100 nM)	30
6,19-Epidioxy-24,24-difluoro-(7E)-9,10-secocholesta-5(10),7-diene-3β,25-diols	ca.10 (10 nM) ca.35 (ca.60 nM) ca.50 (ca. 600 nM)	45
6,19-Epidioxy-(7E,24R)-9,10-secocholesta-5(10),7-diene-3β,25-diols	—	150-200
6,19-Epidioxy-(7E)-9,10-secocholesta-5(10),7-diene-3β,25-diols	ca.5 (10 nM) ca.20 (60 nM) ca.50 (600 nM)	120-130
6,19-Epoxy-(7E,19ξ)-9,10-secocholesta-5(10),7-diene-3β,25-diol	ca.10 (ca.6 nM) ca.35 (ca.60 nM) ca.70 (ca.600 nM)	—
6,19-Epoxy-(7E,19ξ)-9,10-secocholesta-5(10),7-diene-3β,25-diol	ca.10 (ca.6 nM) ca.35 (ca.60 nM) ca.70 (ca.600 nM)	—

*Data estimated from graphed results.[61,62]
†Potencies relative to that of 1α,25-dihydroxy-Vitamin D_3.[61]

lest-5-en-22-one, and 15-ketone *12* was relieved by exogenous cholesterol. It is presumed that de novo sterol biosynthesis inhibition resulting in a diminished ratio of membrane cholesterol to phospholipid thereby decreased cell membrane adhesion properties.[159] A similar case wherein the 3β,25-diol *7* inhibit the Ca^{2+}-dependent aggregation (and subsequent fusion) of cultured chick myoblasts may implicate a compromised local availability of membrane sterol but also may involve effects of membrane Ca^{2+} flux.[160]

These odd in vitro effects of oxysterols thus continue to implicate both de novo sterol biosynthesis and membrane effects in complex manner.

ATHEROGENICITY

One of the more prevalent suggestions of oxysterol biological activity is that of atherogenicity or angiotoxicity. The suspicion that oxysterols be implicated in the origins and progression of human aortal atherosclerosis has been held for over two decades, but the matter still remains uncertain. Demonstrations of oxysterols cytotoxicities, discovery of oxysterols in human aortal tissues and plaques (including recently that of the 5,6-epoxides *5* and/or *8* and the 3β,25-diol *7*[161]), the increased accumulation of 3β,26-diol *11* and its fatty acyl esters with advancing age and severity of atherosclerosis, and identification of the 3β,26-diol *11*, its esters, and related oxysterols in human blood combine to provide a basis for this suspicion.[4,162-167]

Indeed, many of the arguments linking cholesterol to human atherosclerosis may be advanced with equal validity for oxysterols present in foods, blood, and aortal tissues. Were there epidemiological data examining relationships between atherosclerosis or cardiovascular disease with plasma oxysterols levels as independent risk factors, perhaps a more balanced judgement could be approached.[168]

Crucial to the involvement of oxysterols with aortal atherosclerosis is the presence of recognized cytotoxic or atherogenic oxysterols in human blood. Here the evidence is ample that oxysterols (together with their fatty acyl esters) occur at appreciable levels distributed among the plasma lipoproteins. A cholesterol fatty acyl ester hydroperoxide has also been discovered in human blood (317 pmol/mL),[169] and in WHHL rabbit aorta lipids,[170] once again suggesting that such peroxides may exist in blood and tissues.

Among the plasma oxysterols indicated to be the most toxic as well as atherogenic are the 3β,25-diol *7* and the 3β,26-diol *11*, both of which accumulate in the human aorta. The 3β,5α,6β-triol *6* and 3β,26-diol *11* cause extensive necrosis and an acute inflammatory response when implanted subcutaneously in rats,[99] and the triol *6* and 3β,25-diol *7* have been repeatedly identified as the most active oxysterols in a variety of tests related to atherosclerosis.

Despite much attention focused on the cytotoxic properties of oxidized plasma lipoproteins, relatively little concern for the composition of plasma lipoproteins with respect to their oxysterols has developed.[171] Nonetheless, as human plasma contains a variety of

oxysterols that increase in amounts as the plasma ages and also exhibits toxic properties in bioassays, it is not unreasonable to suspect that plasma lipoprotein oxysterols be implicated in such phenomena.

The cytotoxic properties of rat plasma very low density lipoprotein (VLDL) do not appear to be from the 3β,25-diol *7*,[121] but other oxysterols may be implicated. Support for the view has been adduced by the lethal effect of intravenous injection of low density lipoprotein (LDL) previously treated with a microbial cholesterol oxidase into rabbits bearing aortic atheromatous lesions. The enzymically oxidized lipoprotein was also toxic to several cultured cell lines. These results suggest that lipoprotein cytotoxicity may derive from the product 3-ketone *9* formed by enzymic oxidation of lipoprotein cholesterol.[103]

The common natural endogenous oxysterols may distribute differently among the recognized plasma lipoprotein fractions. Analyses of endogenous human plasma oxysterols suggest the 7-ketone *4* and isomeric 5,6-epoxides *5* and *8* be present on balance in LDL, with the 3β,26-diol *11* distributed more evenly (ca. 2:1) between VLDL and LDL on the one hand, high density lipoprotein (HDL) on the other.[13,15] However, exogenous 3β,7α-diol *2*, 3β,25-diol *7*, and 3β,20-diol *10* appear to favor distribution into LDL and HDL of human serum.[41] In squirrel monkeys it appears that exogenous 3β,25-diol *7* is selectively present in VLDL and LDL but in low levels in HDL.[27,29,33,164,165] Rabbit plasma lipoproteins appear to carry the 7-oxygenated sterols *2-4* and the 3β,5α,6β-triol *6* selectively in VLDL, the 3β,25-diol *7* in LDL.[33,34] It remains uncertain what significance, if any, attaches to these distributions.

One of the most active areas of investigation of oxysterols with respect to atherosclerosis is that of the presence of oxysterols in cholesterol-rich processed foods. However, the obvious concern that chronic ingestion of low levels of oxysterols be a constant threat of damage to arteries has yet to be examined satisfactorily by experiment. In one such attempt White Carneau pigeons recognized as susceptible to atherosclerosis were fed for three months a low level of cholesterol with traces of the 3β,5α,6β-triol *6*. Only evidence of damage to coronary arteries was observed.[172]

Toxic actions of oxysterols that may be related to their in vivo atherogenic effects are listed in Table 5 and Table 6. Some of the in vitro observations extrapolated to in vivo cases suggest their relationship to atherogenesis. Thus, suppression of prostaglandin biosynthesis in smooth muscle cells in vivo might lead to

Table 5. In Vivo Cytotoxic and Atherogenic Effects of Oxysterols

Oxysterol	Test System	Results	Reference
3β-Hydroxycholest-5-en-7-one *4*	Chicks fed 10–20 mg/d	Damage to aorta, toxic to smooth muscle cells	173
Cholest-5-ene-3β,25-diol *7*	Squirrel monkeys, fed 5 mg/kg	Intimal thickening; proliferation of smooth muscle cells, edema; deposits of cellular detritus and calcium	174
	New Zealand white rabbits, 2.5 mg/kg intravenously	Aorta surface damage, adhering platelets	175, 176
5α-Cholestane-3β,5,6β-triol *6*	Squirrel monkeys, fed 5 mg/kg/day	Intimal thickening, proliferation of smooth muscle cells, accumulations of collagen fibers, calcium	177
	New Zealand white rabbits, 2.5 mg/kg intravenously	Aorta surface damage, adhering platelets	175, 176
	Wistar rats, *per os*	Damage to aortic smooth muscle cells and epithelium	178
	White Carneau pigeons fed in diet	Coronary artery atheromas	172
Commercial cholesterol (containing oxysterols)	Chinchilla rabbits, fed 200 mg/kg for 10 weeks	Serum cholesterol increased	89
Mixed oxysterols	Japanese quail, 0.5% in diet	Aortic cholesterol increased	179
	Chicks, quail	Early decrease in weight gain; lethal!	92
	White rabbits, 250 mg/kg by gavage	Increased smooth muscle cell death within 24 h	164, 180, 181
	New Zealand white rabbits, 1 g/kg by gavage	Increased death of aortic medial and intimal cells by pyknosis; calcium deposits	182
	Sprague–Dawley rats fed 2% in diet	Lipid peroxidation and prostaglandin formation slightly increased	183

Table 6. In Vitro Oxysterols Effects Related to Atherosclerosis

Oxysterol	Test System	Effects	References
Cholest-5-ene-3β,7-diols *2,3*	Rabbit aorta smooth muscle cells	Cholesterol uptake inhibited 40% at 50 μg/mL	184
3β-Hydroxycholest-5-en-7-one *4*	Human aorta smooth muscle cells	Growth inhibition at 5 μg/mL	146
	Perfused pig carotid arteries	Inhibition of LDL uptake by 22%	185
	Rabbit aorta smooth muscle cells	Cholesterol uptake inhibited 40% at 50 μg/mL	184
	Canine coronary artery cells	Weak constriction effect	186
5,6α-Epoxy-5α-cholestan-3β-ol *5*	Rabbit aorta smooth muscle cells	Cholesterol uptake inhibited 60% at 50 μg/mL	184
5,6-Epoxy-5αβ-cholestan-3β-ols *5,8*	Human aorta smooth muscle cells	Growth inhibition at 5 μg/mL; lysosome activation at 1–10 μg/mL	146
5α-Cholestane-3β,5,6β-triol *6*	Rabbit aorta smooth muscle cells	Prostaglandin biosynthesis inhibited	187
		Albumin transfer across confluent monolayers increased	188
		Cholesterol uptake inhibited 70% by 50 μg/mL, 90% by 100 μg/mL	184
	Canine smooth muscle cells	Toxic at 0.5–6 μg	189
	Rabbit vascular endothelium	Endocytosis decreased	190
Cholest-5-ene-3β,25-diol *7*	Human smooth muscle cells	Growth inhibition at 5 μg/mL, toxic at 10 μg/mL; decreased DNA synthesis at 2 μg/mL; lysosome activation at 1–10 μg/mL	146
	Human skin fibroblasts	Increased HDL binding by 20 μg/mL	120
	Human arterial smooth muscle cells	Increased HDL binding at 20 μg/mL	120
	Bovine vascular endothelial cells	Increased HDL binding at 50–100 μg/mL	191
	Human monocyte J774 macrophages	Decreased LDL receptor activity at 1 μg/mL	192, 193
	Canine smooth muscle cells	Toxic at 1–6 μg	189
	Rabbit aorta smooth muscle cells	Cytotoxic effects	181, 194, 195
		Cholesterol uptake inhibited 60% by 50 μg/mL	194
		Prostaglandin biosynthesis inhibited	187
	Swiss-Webster mouse peritoneal macrophages	Apolipoprotein E synthesis increased 3-fold by 5 μg/mL	156
	White Carneau pigeon aorta smooth muscle cells	Increased LDL binding and sterol esterification; suppressed *de novo* sterol biosynthesis;	196, 197
(25R)-Cholest-5-ene-3β,26-diol *11*	Human skin fibroblasts	Inhibition of LDL uptake, degradation	198
(25S)-Cholest-5-ene-3β,26-diol	Human skin fibroblasts	Inhibition of LDL uptake, degradation	198

increased platelet aggregation and resultant thrombus formation.[187] However, evidence of possible increased prostacyclin production in aortal tissues of rats fed oxysterols is also available.[183] Oxysterol inhibition of albumin transfer between cells suggests that in vivo the capacity of the aorta endothelium to act as a selective permeability barrier to plasma protein might be compromised.[188] These suggestive matters need further consideration.

Again, the 3β,25-diol *7* and 3β,5α,6β-triol *6* have consistently been the most toxic in these several tests.[174,177,181,187,194] The ability of 3β,5α,6β-triol *6* to damage aortal tissue of rats ordinarily resistant to natural and experimental arteriosclerosis emphasizes its toxicity in this regard.[178]

These two oxysterols appear to exert different toxic effects by different mechanisms. For instance, the 3β,5α,6β-triol *6* decreases endocytosis activity in cultured rabbit vascular endothelial cells, but the 3β,25-diol *7* had little effect.[190] The growth inhibitory effects of the 3β,25-diol *7* may not be reversible, but exogenous cholesterol protects partially.[146] Other marked differences between actions of the oxysterol pair suggest that perhaps the 3β,25-diol *7* exert its effects via suppression of HMGCoA reductase, whereas the 3β,5α,6β-triol *6* act by other mechanisms, perhaps via membrane effects.

The indicated capacity of the 3β,25-diol *7* to increase HDL binding in human fibroblasts and arterial smooth muscle cells[120] and that of the isomeric 3β,26-diols *11* to inhibit LDL uptake into human fibroblasts support further a possible role of these endogenous oxysterols in the regulation of cellular cholesterol homeostasis. The effect on LDL uptake of the 3β,26-

diols occurs at oxysterol concentrations (EC_{50} ca. 1 μM) potentially attained in human blood.[198]

In other cells oxysterol effects may be quite different. In J774 macrophages the inhibitory actions of the 3β,25-diol *7* on LDL uptake and HMGCoA reductase suppression are considerably different from those in cultured fibroblasts.[30] However, just the opposite effect of the 3β,25-diol *7* effecting increased LDL binding in aorta smooth muscle cells of the atherosclerosis-susceptible White Carneau pigeon appears the case.[196] In a simple study comparing commercial cholesterol containing oxysterols with purified cholesterol fed Chinchilla rabbits, where serum cholesterol levels rather than aortal damage were compared, higher serum cholesterol levels were attained with the unpurified sample.[89]

Despite these several items evincing both acute and chronic toxic effects of oxysterols associated with aortal atherosclerosis, some evidence suggests otherwise. An impressive study of the comparative effects of pure cholesterol versus cholesterol oxidation products fed rabbits concludes that the oxysterols were less atherogenic than pure cholesterol, thus in direct opposition to the weight of evidence adduced on the topic heretofore. Moreover, a relatively sweeping conclusion was voiced with respect to (presumably human) atherosclerosis, "attention should remain on cholesterol rather than cholesterol oxides."[199,200] It is somewhat surprising that this one recent report that oxysterols not be atherogenic agents be so rapidly and broadly accepted in the generalized sense. Several ad hoc statements that oxysterols cannot be primary factors in atherosclerosis have appeared.[64,103,201]

Carefully purified, analyzed, and stored cholesterol was fed female New Zealand white rabbits for 11 weeks at 166 mg/kg/day and aortal lesions compared with those from animals fed cholesterol-free mixed oxysterols generated by oxidizing cholesterol by air in refluxing toluene. The lesions of the cholesterol-fed group were more severe by macroscopic and microscopic pathology evaluations than those from groups fed oxysterols or cholesterol-oxysterols mixtures.

Reconciliation of these results with those of others is not now possible. The different oxysterols composition of the mixtures employed, the different doses administered, and the possibility of synergism in one or the other study may all be implicated. The most obvious item that sets the study apart is the means by which cholesterol was oxidized and the absence of the highly toxic 3β,5α,6β-triol *6* from the oxysterols mixture.

Although cholesterol is oxidized in refluxing toluene and the common oxysterols are formed, the oxysterols preparation is subtly different from that derived by air-aging of cholesterol. It may also be noted that although [^{3}H]-cholesterol preparations be analytically pure, for some purposes biological discrimination may occur among different samples.[202,203] Whether traces of oxysterols are implicated or whether other effects are involved, these instances suggest caution in hasty generalizations drawn from study of any one cholesterol or oxysterol batch.

In another relevant study hybrid hares (*Lepus europaeus* male × *Lepus timidus* female) fed 1% cholesterol or 1% cholesterol with 0.5 mg/day 3β,25-diol *7* for 7–14 months developed aortal lesions, with no apparent effect of the 3β,25-diol *7* noted.[204]

Yet another viewpoint anent oxysterols involvement in human atherosclerosis has been advanced by Krut. The well known solubilizing effects of oxysterols on cholesterol if applicable in vivo might actually solubilize cholesterol from tissue deposits, including those of aortal lesions. In concept, absorbed dietary oxysterols once present in greater abundance in foods less protected from spoilage may have been beneficial in dissolving cholesterol from developing plaques or lesions, but with more modern food processing that reduces the amount of oxysterols in foods, the once protection now be less. Accordingly, cholesterol deposits are not removed as well, thus increasing the incidence of aortal lesions and atherosclerosis.[205–208] Some support of this viewpoint is had in the recent report that white rabbits fed oxysterols (50 mg/day) from air-aged USP cholesterol appear to protect against formation of aortal lesions.[64,209]

Although these ideas are not readily reconciled with other viewpoints summarized here, cholesterol-rich foods unprotected from autoxidation do accumulate higher levels of oxysterols, and more mild processing or storage methods appear to reduce oxysterols levels. Whether beneficial or no, diminished oxysterols levels in foods is now of concern to the food industry, but it is obvious that subtle factors not now recognized be implicated in the dietary oxysterol issue.

The monoclonal hypothesis of Benditt suggesting that aortal lesions are monotypic, arising from a single cell in which the 5α,6α-epoxide *5* might be the initiating injurious agent,[210] may yet be more seriously implicated in atherogenesis, as the mutagenic and transforming capacities of 5α,6α-epoxide *5* (cf. the ensuing section of this review) and other blood oxysterols be demonstrated. The presence of the 5,6-epoxides *5* and *8* in human aortal tissue[161] and blood (partitioned into VLDL, LDL, and HDL)[13] would support such formulation. However, although mutagens and cytotoxic components have been found in human

VLDL, the activity does not come from oxysterols such as the 3β,25-diol *7*,[121] and detection of aortal tissue mutagens by the Ames test has not been feasible.[211,212] By contrast, mitogenic activity from human aorta tissue has been detected by bioassay,[213] but the activity is not from lipids.

Furthermore, an alternative concept of selective survival of one phenotype over another may account for the existence of monotypic aortal lesions. Hybrid *Lepus europaeus* × *Lepus timidus* hares with glucose 6-phosphate dehydrogenase isozyme mosaicism in females, fed 1% cholesterol with or without supplemental 3β,25-diol *7*, develop hypercholesterolemia and aortal lesions.[204,214] More interestingly, hybrid hares fed 1% cholesterol plus 3β,25-diol *7* or 3β,5α,6β-triol *6* (but not in hares fed 1% cholesterol alone) developed monotypic aortal lesions in which the *timidus* phenotype predominated, with similar effects in aorta tissue without lesions.[214-216]

The ratio of *europaeus* phenotype to *timidus* phenotype in cultured hare skin fibroblasts appears to be shifted by exogenous 3β,25-diol *7* or by ultraviolet light irradiation in favor of the *europaeus* phenotype, perhaps because the *timidus* phenotype be more sensitive to toxic effects.[217-219] The differential survival of the *europaeus* phenotype in vitro but of the *timidus* phenotype in vivo establish that major differences between toxic effects in vitro and in vivo occur! These results tend to modify the monoclonal hypothesis of Benditt but demonstrate the power of dietary oxysterols to produce subtle tissue effects perhaps not so easily recognized in less well designed studies.

One of the most impressive arguments that strongly implicates oxysterols as atherogenic agents comes from epidemiologic study of Indian immigrants in London who are afflicted with higher than expected mortality from atherosclerosis but who are not characterized by the usual high risk factors associated with such disease frequency. These people continue to consume large amounts of ghee, the clarified butter used extensively for Indian cooking and which contains up to 12.3% (!) oxysterols not found in fresh butter. The oxysterols of ghee include the 3β,7-diols *2* and *3*, one or both 5,6-epoxides *5* and/or *8*, the 3β,25-diol *7*, and the 3β,20-diol *10*. An obvious suggestion arises that it be the oxysterols of ghee that may cause the high incidence of human atherosclerosis.[220]

Clearly the definitive concept about the relationship of oxysterols to human atherosclerosis has not surfaced.

MUTAGENICITY AND CARCINOGENICITY

It is advantageous to discuss developments in studies of oxysterol mutagenicity and carcinogenicity together, as there are now reliable demonstrations of both biological activities for specific oxysterols where heretofore there were uncertainties. Cholesterol is neither mutagenic nor carcinogenic, and recent tests for transformation activity in cultured Syrian hamster embryo[221,222] and HeLa cells[49,50] confirm this inactivity. However, it may be noted that enteric bacteria appear to catalyze binding of cholesterol to calf thymus DNA in vitro,[223] and heating cholesterol with creatine may create mutagenic products,[224] which if formed in heated milk are not detected.[225]

Moreover, autoxidized cholesterol preparations rich in polar oxysterols are mutagenic towards *Salmonella typhimurium* TA1537, TA1538, and TA98 test strains in the Ames test, but individual mutagenic oxysterols have not been identified.[51,59] Oddly, neither autoxidized brassicasterol, cholesta-5,7-dien-3β-ol, ergosterol, lanosterol, sitosterol, nor stigmasterol were mutagenic.[51]

Demonstration of mutagenicity for specific oxysterols has not been successful.[51,101,226] Similar negative results are regularly reported for bile acids on the standard agar plate bioassays[226] and uncertain results in liquid incubation bioassays.[227-229] However, N-nitroso derivatives of glycocholic or taurocholic acids are mutagenic in a forward mutation bioassay with *S. typhimurium* TM677.[230] The co-mutagenic effect of bile acids towards strains of *S. typhimurium*[231] and their promoting effect on transformation of fibroblasts in vitro[232] suggest the more highly oxidized sterol derivatives also be toxic agents.

It is not clear whether the routine agar plate bioassay is adequate, as the test organism may be sensitive to unrelieved analyte toxicity and physical phase separation of analyte from homogeneous dispersions or vehicle may interfere with bacterial respiration. As no means of adjusting these toxic effects of bacterial survival and growth exist for the agar plate bioassay, the use of liquid incubation methods that allow adjustment of toxicity has expanded, and autoxidized cholesterol exhibits greater mutagenicity in such bioassay.[59]

Furthermore, the mutagenicity of specific oxysterols in both bacterial and cultured mammalian cell systems has now been demonstrated. By such means a weak dose-response mutagenicity for the initial cholesterol oxidation products cholesterol 7α-hydroperoxide (from $^3O_2$) and 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (from $^1O_2$) has been demonstrated using *S. typhimurium* TA1537. Both sterol hydroperoxides exhibited toxicity towards the test organism, and phase separation of analyte occurred at the higher test doses. Moreover, the test strain metabolized the sterol hydroperoxides. The 7α-hydroperoxide was transformed to the 7-ketone *4* and reduced to the corresponding 3β,7α-diol *2*, both being nonmutagenic. However, the

5α-hydroperoxide was isomerized under enzyme catalysis to the 7α-hydroperoxide, thus to a mutagenic species, as well as transformed to the 7-ketone *4* and reduced to 5α-cholest-6-ene-3β,5-diol, these complications mitigating proper assessment of the true mutagenicity of the sterol hydroperoxides.[59,60]

Additionally, the sterol hydroperoxides may not be mutagenic per se, as exogenous catalase abolished but exogenous superoxide dismutase stimulated the mutagenic responses. These results implicate active oxygen species as mutagens, superoxide dismutase generating hydrogen peroxide from superoxide that must have been formed in one-electron reduction of oxygen stimulated by the sterol hydroperoxide analytes. Hydrogen peroxide then be the ultimate mutagen, the action of which is abolished by added catalase.[59] Hydrogen peroxide is weakly mutagenic towards *S. typhimurium* TA100[233,234] and TA102,[235-237] but induces catalase in several strains[234,238] as well as numerous other proteins, five of which are also induced by heat shock.[239] In that cumene hydroperoxide also mutagenic towards *S. typhimurium* likewise induces catalase and other proteins,[238,239] the same process may hold for the mutagenic sterol hydroperoxides.

The scheme of Figure 1 summarizes these results and suggests that the sterol hydroperoxides stimulate one-electron reduction of dioxygen. Other interpretations are possible, for example, if sterol hydroperoxide and hydrogen peroxide interact to form a mutagen. Erratic bioassay data obtained in bioassay-directed search of very polar cholesterol autoxidation products for active mutagens[51] may also have involved generation of hydrogen peroxide or superoxide as the mutagenic species actually detected.

The metabolic transformation of the 5α-hydroperoxide to the 7-ketone *4* directly without prior isomerization of the 5α-hydroperoxide to the 7α-hydroperoxide suggests that sterol peroxyl and oxyl radicals be implicated, as we have seen also in model chemical systems.[240] Whether such oxygen radicals also be mutagenic has not been addressed.

Although sterol hydroperoxides have not been identified in vivo, a cholesterol 3β-linoleate hydroperoxide has been detected in human blood plasma.[169] If the steryl ester hydroperoxide be an endogenous metabolite not generated during analysis, its presence in blood leaves open the possibility that such peroxides affect in vivo toxicity. Indications are that human aortal lip-

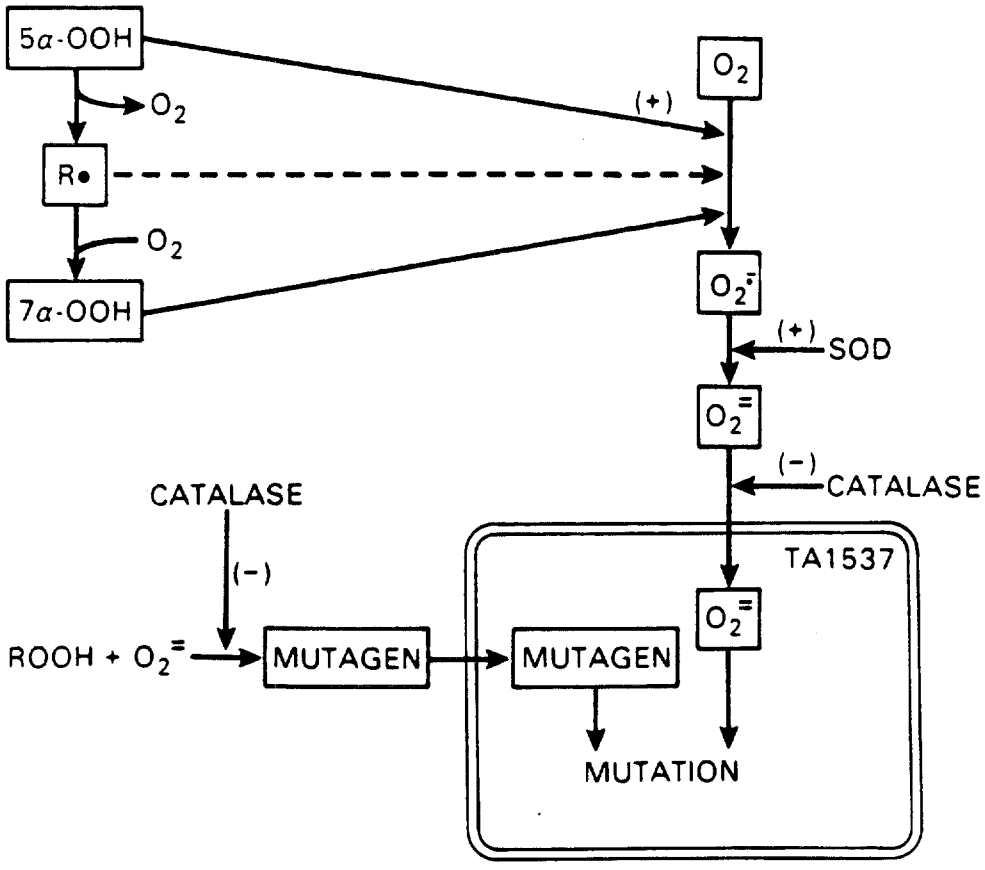

Fig. 1. Processes implicated in the mutagenicity of sterol hydroperoxides. Abbreviations: 5α-OOH, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide; 7α-OOH, cholesterol 7α-hydroperoxide; R·, intermediate sterol radicals; SOD, superoxide dismutase; ROOH, sterol hydroperoxide; TA1537, *S. typhimurium* TA1537.

ids[211,212] and plasma very low density lipoproteins[241] may accumulate mutagens detectable by the bacterial bioassay, but it is uncertain whether such mutagens be oxysterols, oxidized unsaturated lipids, or xenobiotic mutagens.[242]

The 5α,6α-epoxide *5* has been a suspected carcinogen for decades and has been repeatedly tested with the routine agar plate Ames test using several test strains of *S. typhimurium*, all results being negative.[51,243] Moreover, the inhibition of DNA synthesis in HeLa cell cultures by the 5α,6α-epoxide *5* shown in earlier bioassay has since been recognized as from an impurity in the sample tested.[49,50] Nonetheless, the mutagenicity of the 5α,6α-epoxide *5* has been demonstrated in V79 Chinese hamster lung fibroblasts.[46] The V79 fibroblasts accumulated exogenous 5α,6α-epoxide *5* and transformed it to nonmutagenic 3β,5α,6β-triol *6* during bioassay, but direct acting mutagenicity was observed. The isomeric 5β,6β-epoxide *8* was even more mutagenic than the 5α,6α-epoxide *5*.[47]

Both isomeric 5,6-epoxides *5* and *8* are subject to enzymic hydrolysis to the nonmutagenic but more toxic 3β,5α,6β-triol *6*, the greater the hydrolysis the lower the mutagenic response but the greater the toxicity provoked. Both 5α,6α-epoxide *5*, 5β,6β-epoxide *8*, and 3β,5α,6β-triol *6* were cytotoxic and inhibited thymidine incorporation into DNA (3β,5α,6β-triol *6* > 5β,6β-epoxide *8* > 5α,6α-epoxide *5*).[46,47,244,245] The 5β,6β-epoxide *8* has also been shown to be active in a sister chromatid exchange bioassay using cultured human lymphocytes, whereas the isomeric 5α,6α-epoxide *5* and 3β,5α,6β-triol *6* were not.[246] Bioassay results are thus the sum of the several metabolic and toxicity effects.

Older claims of carcinogenicity of cholesterol 5α,6α-epoxide *5* in treated test animals have generally been discounted for want of reliable supporting evidence. What little experimental work that has been reported (liver tumors and abnormal spleens in $B6C3F_1$ mice fed 1% 5α,6α-epoxide *5* in the diet for 6 weeks) leaves the matter unsettled.[82] Furthermore, the hypothesis that the 5α,6α-epoxide *5* be a carcinogenic agent formed in situ in skin exposed to irradiation is also uncertain. Generation of 5,6-epoxides *5* and *8* is dependent on the amount of irradiation and on resident antioxidant levels,[247,248] and the cutaneous levels of 5,6-epoxides formed do not approach those required to cause tumors in mice.[154,249] Moreover, the precise composition of the cholesterol 5,6-epoxides formed in irradiated skin has not been determined. Accordingly, whether the 5α,6α-epoxide *5* be carcinogenic in this setting remains uncertain.

However, the suspected carcinogenicity of the 5,6-epoxides *5* and *8* may have a more sound basis in the previously described mutagenicity tests and in recent studies of cell transforming actions of these oxysterols. The 5α,6α-epoxide *5* and its metabolite or hydrolysis product 3β,5α,6a-triol *6* both exhibited transforming activity against cultured Syrian hamster embryo cells.[109,221,222] Additionally, both isomeric 5,6-epoxides *5* and *8* exhibited transforming activities against cultured V79 Chinese hamster lung fibroblasts and C3H-10T1/2 mouse embryo cells, the 5β,6β-epoxide *8* being the more potent. The extent of cell transformation increased with analyte concentration and with exposure time.[48] In that cell transforming activity may be taken as indication of carcinogenicity, these data now suggest that both 5,6-epoxides and their common hydrolysis product 3β,5α,6β-triol *6* be carcinogenic!

The repeated discovery of the cholesterol 5,6-epoxides and the 3β,5α,6β-triol *6* in human tissues associated with cancer further supports this viewpoint. Human breast fluid has been shown to contain mutagens detected with *S. typhimurium* TA1538[246,250] and fluctuating levels of both 5,6-epoxides and 3β,5α,6β-triol *6*, the 5β,6β-epoxide *8* predominating.[251,252] Human prostate gland secretions likewise contain both 5,6-epoxides, in this case the ratio of 5α,6α-epoxide *5* to 5β,6β-epoxide *8* being 10:1.[253] Both 5,6-epoxides *5* and *8* have been measured in human blood as well.[13]

The general concept that oxysterols may act as "alkylating" agents has been tested with model systems involving the oxidation of the dienols ergosterol and cholesta-5,7-dien-3β-ol by I_2 or by $FeCl_3/H_2O_2$, proposed as mild oxidation systems "physiologically similar" to in vivo systems. The reactive oxysterols formed could be intercepted by 1-methylimidazole, suggesting such processes as possible ones implicated in oxysterol mutagenicity and/or carcinogenicity.[254]

These several studies add new support to the viewpoint that cholesterol hydroperoxide and epoxide derivatives be mutagenic and carcinogenic, but in vivo conclusive tests of the proposition remain to be conducted.

MEMBRANE EFFECTS

The importance of cholesterol to the stability and function of cellular membranes is well established; thus, it is not surprising that oxysterols affecting sterol metabolism exert significant effects on plasma membranes of cultured cells and on synthetic membranes in model systems. As in all in vitro study of dispersed cholesterol, it must be noted that cholesterol is subject to oxidations occurring during study,[255,256] with both radical autoxidation and photosensitized oxygenation

processes generating oxysterols that may affect results.[256-260]

The incorporation of exogenous oxysterols into plasma membranes of cultured cells has been repeatedly demonstrated. In the membrane insertion hypothesis it is envisaged that exogenous oxysterol be incorporated into membranes, displacing membrane cholesterol, with attendant effects on membrane stability and function. Indeed, in cultured cells such membrane oxysterols may be a continuing source of intracellular oxysterol potentially influencing intracellular events. Demonstration of such effects in vivo has yet to be addressed.

As examples, human erythrocytes absorb the 7-ketone *4*, cholesta-4,6-dien-3-one, or 1-methyl-19-norcholesta-1,3,5(10)-trien-3β-ol from phospholipid liposomes[261] and the 3β,7-diols *2* and *3*, the 3β, 20-diol *10*, and 3β-hydroxycholest-5-en-22-one from lipoprotein-depleted medium.[41,262] Pig aortal tissues incubated in human plasma enriched with the 7-ketone *4* also absorb the oxysterol.[263]

Incorporation of exogenous oxysterols into plasma membranes may alter cell morphology, survival, growth, and function. Morphological changes observed by light and electron microscopy include microvilli development in rat Morris hepatoma HTC cells treated with the 3β,7β-diol *3*,[58] deformations of bovine platelets and erythrocytes by the 3β,7β-diol *3* or (22R)-cholest-5-ene-3β,22-diol,[41,264] degeneration of human glioma cells by the 3β,25-diol *7*,[122] and loss of synchrony in beating of cultured chick myocardial cells.[116] Sterol distribution within mouse 3T3 cells following administration of the 3β,25-diol *7* is readily visualized.[265]

The survival of cultured cells in the presence of oxysterols is greatly compromised, and many instances appear to involve membrane phenomena (Table 2). The incorporation of oxysterols into plasma membranes may not support growth; neither absorption of cholest-5-en-3-one into human macrophage-like U937 cell membranes[266] nor of several oxystyerols into *Mycoplasma gallisepticum* membranes[104] supported cell growth.

The cytotoxic effects of closely related oxysterols may be quite different; (22R)-cholest-5-ene-3β,22-diol and epimeric (22S)-cholest-5-ene-3β,22-diol are incorporated into erythrocytes equally, but the (22R)-3β,22-diol is much more effective in lysing cells.[158,264,267] Correlated with the diminished toxicity of the (22R)-3β,22-diol relative to the (22S)-3β,22-diol epimer is diminished inhibitory power in suppressing DNA and HMGCoA reductase activity,[137] although it remains uncertain how membrane effects are related to these nuclear events. The differential lytic effect of the epimeric 3β,22-diols on cultured cells may derive from the decreased ability of the (22R)-3β,22-diol to interact with membrane phospholipid to form and maintain a stable and functional membrane. Although the 3β,20-diol *10*, (22S)-cholest-5-ene-3β,22-diol, (23R)-cholest-5-ene-3β,23-diol, (23S)-cholest-5-ene-3β,23-diol, 3β,25-diol *7*, and 3β-hydroxycholest-5-en-22-one appear to interact like cholesterol with phospholipid in model systems to form a stable membrane system, the (22R)-3β,22-diol does not, presumably because of the altered stereochemistry of the side-chain imparted by the (22R)-22-hydroxyl configuration.[268-271]

Oxysterol insertion into membranes affects membrane protein and phospholipid structure. Incorporation of the 3β,7α-diol *2* into the human erythrocyte membrane results in an increase in protein helical structure, whereas the 3β,20-diol *10* causes increased immobilization of fatty acyl chains within the membrane. Both synergism and antagonism between cholesterol and oxysterol may occur in control of membrane protein and phospholipid fatty acyl chain stereochemistry.[262,271,272]

The 7-oxygenated oxysterols 3β,7-diols *2* and *3* and 7-ketone *4* have a diminished capacity to condense (to increase packing order of) phospholipid fatty acyl chains in phospholipid liposomes,[271,273] but the 3β,20-diol *10*, (22S)-cholest-5-ene-3β,22-diol, the epimeric cholest-5-ene-3β,23-diols, and 3β,25-diol *7* condense these phospholipid bilayers in the manner of cholesterol.[268,271,273] By contrast, in phospholipid monolayers the 3β,7-diols *2* and *3* and the 7-ketone *4* exert a condensing effect, but the 3β,25-diol *7* does not.[274]

These effects in liposomes (and presumably in cellular membranes) may arise from fundamental effects of oxysterol in reducing the temperature and enthalpy for the gel to liquid crystal and bilayer to hexagonal phase transitions. The effects depend on the specific oxysterol and its amount as well as composition of the phospholipid implicated.[268,270,272-276] The resultant effect is that of decreased membrane fluidity,[273,277] with attendant altered membrane permeability and decreased stability.

Oxysterols may also be formed in plasma membranes in situ, as oxidation of membrane cholesterol by extracellular oxidants may occur. Indeed, membrane sterols may serve as antioxidant protection in some cases.[278] Moreover, the 3-ketone *9* may be inserted into phospholipid liposomes and plasma membranes of erythrocytes and cultured fibroblasts by in situ oxidation of membrane cholesterol by cholesterol oxidase.[279-283] The uptake of phosphate anion by human erythrocytes in vitro is inhibited by such treatment,[284] and erythrocyte hemolysis is promoted.[285,286] Human

low density lipoprotein oxidized by cholesterol oxidase (presumably forming 3-ketone *9*) becomes toxic to cultured human cells in vitro and lethal to rabbits upon injection.[103]

In some cases the stability of plasma membranes may be improved by treatment with oxysterols. The resistance of cultured mammalian cells to the cytolytic action of streptolysin O is increased by 3β,25-diol *7* or 3β,20-diol *10*, possibly by lowering plasma membrane cholesterol accessible to the toxin.[287] By contrast, in vitro uptake of oxysterols into the erythrocyte plasma membrane causes an expansion of cell surface area without attendant cell volume changes, thus a diminution of osmotic fragility of the erythrocyte. Such protection is accorded by 25 μM 3β,7α-diol *2*, 3β,7β-diol *3*, the 7-ketone *4*, and the 3β,20-diol *10* but also by cholest-5-ene-3β,4β-diol, 5α-cholestane-3β,6β-diol, 3β-hydroxycholest-5-en-22-one, 3β-hydroxy-5α-cholestan-6-one, and 3β,5-dihydroxy-5α-cholestan-6-one. The more oxysterol inserted into the membrane, the greater the protection, but serum lipoprotein diminished the effect.[288] Erythrocytes from patients with hereditary spherocytosis also respond in vitro to the stabilizing influence of oxysterols on osmotic fragility, suggesting possible future directions for pharmacologic approach to management of this blood disorder.[288,289]

Media composition greatly influences these in vitro results. Thus, oxysterol uptake into cultured human lymphocytes is decreased in media containing lipoproteins. Lipoproteins also are able to remove oxysterols from lymphocyte and erythrocyte membranes[261,288] and from phospholipid-oxysterol monolayers.[275]

Several cellular membrane functions influenced by oxysterols, including sugar uptake and membrane Na^+/K^+-ATPase activity (Table 7), must pose a profound stress on cell viability. In these data a clear distinction may be made between the actions of the toxic oxysterols 3β,5α,6β-triol *6* and 3β,25-diol *7*, the 3β,5α,6β-triol *6* affecting hexose uptake, the 3β,25-diol *7* not.[127,287,291–294] It is reasoned that the 3β,5α,6β-triol *6* insert into the plasma membrane, whereas the 3β,25-diol *7* exert its toxic effects via reduction in de novo sterol biosynthesis.[127,291–294]

Table 7. Membrane Effects of Oxysterols

Oxysterol	Membrane System	Effects	Reference
Cholest-5-ene-3β,7α-diol *2*	Phospholipid liposomes	Decreased glucose permeability	275
Cholest-5-ene-3β,7β-diol *3*	Phospholipid liposomes	Decreased glucose permeability	275
3β-Hydroxycholest-5-en-7-one *4*	Perfused pig carotid artery	Diminished uptake of LDL	185
	Phospholipid liposomes	Decreased glucose permeability	275
5,6α-Epoxy-5α-cholestan-3β-ol *5*	Dog brain synaptosomes	Na^+/K^+-ATPase (EC 3.6.1.3) stimulated by ca.1.5 μM	290
5α-Cholestane-3β,5,6β-triol *6*	Rabbit aorta smooth muscle cells	Hexose transport decreased by 5–50 μg/mL	127, 291–294
		5'-Nucleotidase (EC 3.1.3.5) inhibited	127, 293–298
		Na^+/K^+-ATPase inhibited	127, 293–298
Cholest-5-ene-3β-19-diol	Dog brain synaptosomes	Na^+/K^+-ATPase stimulated by ca.1.5 μM	290
(20S)-Cholest-5-ene-3β,20-diol *10*	Dog brain synaptosomes	Na^+/K^+-ATPase stimulated by ca.1.5 μM	290
(22R)-Cholest-5-ene-3β,22-diol	Phospholipid liposomes	Dye permeability increased	268
(22S)-Cholest-5-ene-3β,22-diol	Monkey artery smooth muscle cells	LDL receptor sites diminished	299
	Dog brain synaptosomes	Na^+/K^+-ATPase stimulated by ca.1.5 μM	290
	Phospholipid liposomes	Dye permeability decreased	268
25-Fluoro-(22S)-cholest-5-ene-3β,22-diol	Monkey artery smooth muscle cells	LDL receptor sites diminished	299
Cholest-5-ene-3β,25-diol *7*	Rabbit aorta smooth muscle cells	5'-Nucleotidase inhibited	127, 293–298
		Na^+/K^+-ATPase inhibited	127, 293–298
	Bovine vascular endothelial cells	HDL binding sites increased 5- to 10-fold by 10–100 μg/mL	191
	Human fibroblasts	LDL uptake and degradation decreased	191, 193
	J774 Macrophages	LDL uptake decreased	193
	Phospholipid liposomes	Decreased glucose permeability increased	275
(25R)-Cholest-5-ene-3β,26-diol *11*	Human fibroblasts	LDL uptake and degradation decreased	191, 193
(25S)-Cholest-5-ene-3β,26-diol	Human fibroblasts	LDL uptake and degradation decreased	191, 193

Intracellular functions may also be moderated by oxysterol effects on membranes. The incorporation of the 3β,7β-diol *3* into cultured rat hepatoma cells decreases membrane fluidity and also DNA biosynthesis and HMGCoA reductase activity, whereas the water-soluble 3β,7β-diol *3* 3β,7β-dihemisuccinate does not alter membrane fluidity nor HMGCoA reductase activity but does decrease DNA biosynthesis. These results suggest that DNA biosynthesis not depend on membrane fluidity but that HMGCoA reductase activity does.[277] Attention to correlations of oxysterol insertion into membranes and attendant effects on fluidity with HMGCoA reductase activity, DNA biosynthesis, and other intracellular functions is much needed.

The effects of oxysterols acting on cell membranes may involve yet another fundamental process, that of Ca^{2+} metabolism. Oxysterols modulate Ca^{2+} influx and efflux in cultured cells apparently by being incorporated into cell membranes. Indeed, toxicities of the isomeric 5,6-epoxides *5* and *8* and 3β,5α,6β-triol *6* on endothelial cells, V79 lung fibroblasts, and hepatocytes are significantly reduced in cultures grown in Ca^{2+}-deficient media.[300] However, more than one mechanisms for Ca^{2+} effects may exist.

The implication of intracellular Ca^{2+} as an intracellular "second" messenger of extracellular stimuli is indicated here. In human erythrocytes (with blocked Ca^{2+}-ejecting ATPase) Ca^{2+} influx and efflux is controlled by adjustment of the ratio of membrane cholesterol to phospholipid, a ratio above 1.4 giving increased Ca^{2+} influx, a ratio below 0.75 causing a decreased influx. Similar variations may be imposed by oxysterols, Ca^{2+} influx being increased by the 3β,5α,6β-triol *6*, (22S)-cholest-5-ene-3β,22-diol, 3β,5-dihydroxy-5α-cholestan-6-one, and 3β-hydroxy-5α-cholestan-7-one but decreased by the 3β,7β-diol *3*, 7-ketone *4*, 3β,25-diol *7*, and 3β,20-diol *10*.[301-304] In rat hepatocytes and platelets the 7-ketone *4*, 3β,5α,6β-triol *6*, and 3β,26-diol *11* stimulate Ca^{2+} influx, but Ca^{2+} efflux is increased by the isomeric 5,6-epoxides *5* and *8*.[47,305] The 3β,25-diol *7* increases Ca^{2+} uptake into cultured murine P815 mastocytoma cells.[150]

These results appear to derive from incorporation of oxysterol into the plasma membrane, thereby affecting membrane cholesterol content, membrane fluidity, and the associated Ca^{2+} channels.[306,307] The same kinds of effects may be had in phospholipid liposomes, where uptake of Ca^{2+} is also affected by oxysterols, the 3β,25-diol *7*, 3β,26-diol *11*, and 25-hydroxy Vitamin D_3 all increasing Ca^{2+} uptake into liposomes. Uptake of other divalent cations Mn^{2+}, Mg^{2+}, Sr^{2+}, and Ba^{2+} is also increased by the 3β,25-diol *7*.[305,308]

Physical chemical investigations of artificial bilayer lipid membranes suggest that incorporation of oxysterols into the membrane affects the potential energy barrier to inorganic ion conduction through the membrane as well as membrane fluidity and molecular packing characteristics.[309] Factors influencing ion conductance in the "black lipid membrane" model (containing oxidized cholesterol) include a negative surface charge[310] and an increase in elasticity caused by Ca^{2+}, possibly adsorbed to the membrane oxysterols by a chelation effect.[311] The conductivity of such membranes, including formation of channels or pores, may be influenced by exogenous components that may or may not penetrate the membrane or be incorporated into it.[312,313]

Oxysterols may interact with fatty acyl groups in other ways as well. Thus, cholesterol and cholest-5-ene-3β,4β-diol form cylindrical "myelin" tubes with sodium oleate aqueous solutions but the 3β,25-diol *7*, which has lost the amphiphilic character of the sterol molecule, does not.[314]

Another mechanism by which oxysterols (cholesterol 25-hydroperoxide) may affect Ca^{2+} metabolism is via inhibition of the protein calmodulin implicated in a variety of Ca^{2+}-dependent events.[64,209] This inhibition would have the effect of suppressing actions of Ca^{2+} on intracellular processes, including the Ca^{2+}-calmodulin kinase inhibiting HMGCoA reductase and thereby de novo sterol biosynthesis.[315-317] This result coupled with the other influences of oxysterols on membrane function in Ca^{2+} metabolism cited here pose alternative mechanisms by which HMGCoA reductase activity be regulated.

EFFECTS ON SPECIFIC ENZYMES

Oxysterols affect the activity of several specific enzymes implicated in sterol metabolism, including HMGCoA reductase, acyl cholesterol: acyl coenzyme A O-acyltransferase (ACAT), the cholesterol side-chain cleavage cytochrome P-450_{scc} system, cholesterol 7α-hydroxylase, cholesterol 5,6-epoxide hydrolase, and less well characterized 4- and 14-methylsterol oxidases. The cytoplasmic enzymes acetoacetyl coenzyme A thiolase, HMGCoA synthase, and mevalonate kinase also implicated in sterol biosynthesis are likewise affected by oxysterols. Both inhibition and stimulation are observed, and different oxysterols may have opposing actions in the same system. However, the matter may be one in which an oxysterol influence on an enzyme be an effect on another process that then affects the enzyme activity. A considerable awareness of sterol biochemistry must be had to delve into these several complexities.

3-Hydroxy-3-methylglutaryl Coenzyme A Reductase (EC 1.1.1.34)

By far the one enzyme of major interest is endoplasmic reticulum HMGCoA reductase implicated as regulatory enzyme in de novo sterol biosynthesis. The reductase is a transmembrane glycoprotein that transforms 3-hydroxy-3-methylglutaryl coenzyme A into mevalonate by a four-electron reduction and is subject to rapid regulation by phosphorylation[315-317] and to a slower multivalent feedback regulation.[318-320]

The reductase, together with cytosol acetoacetyl coenzyme A thiolase, HMGCoA synthase, mevalonate kinase, ACAT, LDL receptor synthesis, and squalene synthase, is intimately involved in maintenance of cellular cholesterol homeostasis. The two recognized sources of external plasma lipoproteins and internal de novo sterol biosynthesis for maintaining cholesterol homeostasis plus the variability of tissue specific cholesterol biosynthesis[321] presents a complex arena of regulatory processes yet to be sorted into definitive description.

The rate of de novo sterol biosynthesis seems inversely proportional to plasma lipoprotein availability, with LDL cholesterol and oxysterols implicated as inhibitors of HMGCoA reductase activity[53,322-332] as well as of three other cholesterogenic enzymes acetoacetyl coenzyme A thiolase,[333-335] mevalonate kinase,[333] and HMGCoA synthase (EC 4.1.3.5)[333-341] However, oxysterol effects on HMGCoA reductase activity in arthropods (tobacco hornworm *Manducca sexta*, fruitfly *Drosophila melanogaster*) have not been demonstrated.[242,243]

Two major interests devolve on these matters: 1) discovery of oxysterol chemotherapeutic agents useful for decreasing viability and growth of tumor cells in treatment of cancer and for suppression of de novo sterol biosynthesis potentially lowering plasma cholesterol levels with respect to present concerns about cardiovascular disease and 2) discovery of the means by which oxysterols (or cholesterol) act in regulating the activity of HMGCoA reductase in vitro.

Oxysterol synthesis programs have been mounted for potential chemotherapeutic agents; synthesis of antitumor oxysterols has been mentioned in the Cytotoxicity section of this review. Chemical synthesis of oxysterols as inhibitors of HMGCoA reductase activity is centered around nuclear-substituted oxysterols, particularly C_{27}-sterol 15-ketone derivatives,[344-360] but side-chain substituted derivatives have also been prepared.[299,361]

These actions of oxysterols in suppressing de novo sterol biosynthesis and HMGCoA reductase activity in divers cultured cells are summarized in Table 8 and Table 9, and comparison of both actions in mouse fibroblasts (L cells) is made in Table 10. Although most

Table 8. Oxysterols Inhibition of De Novo Sterol Biosynthesis

Oxysterol	Cell System	Reference
Cholest-5-ene-3β,7β-diol *3*	Guinea pig lymphocytes, 20 μg/mL	362
3β-Hydroxycholest-5-en-7-one *4*	Human peripheral blood mononuclear cells	139
	Human jejunum enterocytes, ca.50% inhibition at 20 μM	363
	MRC-5 Lung fibroblasts, 50% inhibition by 1.5 μg/mL	364
Cholest-5-ene-3β,25-diol *7*	Human hairy cell leukemia, human peripheral blood mononuclear cells, HD_{50} 0.08–0.5 μM; lymphocytes	139, 140, 365
	Human artery smooth muscle cells	127
	Human bone marrow granulocytic progenitor cells, 89% inhibition by 1 μM	118
	Human fibroblasts, 1.25 μM	119, 366
	Human keratinocytes	366
	Monkey artery smooth muscle cells	147, 148
	Rabbit ileal mucosa segments, 0.05–0.5 mM	367
	Guinea pig lymphocytes, 20 μg/mL	362
	Murine myoblasts	145, 160
	Mouse L cells, 88% inhibition by 0.25 μg/mL	287
	Swiss 3T3 mice cells, 10–50 nM	147, 265
	Myoblasts, 1 μg/mL	160
	Epicutaneous application to Hr/Hr hairless mice	368
(20S)-Cholest-5-ene-3β,20-diol *10*	Mouse L cells, 88% inhibition by 0.50 μg/mL	287
Cholest-5-ene-3β,22-diol	Human skin fibroblasts	369
5α-Chyolestane-3β,5,6β-triol *6*	Human artery smooth muscle cells	127
Cholesta-5,7,9-trien-3β-ol	MRC-5 Lung fibroblasts, 50% inhibition by 2.5 μg/mL	364
5α-Lanost-8-ene-3β,25-diol	IEC-6 Rat intestinal epithelial cells, ID_{50} ca.0.1 μM	370
5α-Lanost-8-ene-3β,32-diol	Mouse fibroblasts	371
25-Hydroxy-5α-lanost-8-en-3-one	IEC-6 Rat intestinal epithelial cells, ID_{50} ca.0.5 μM	370
3β-Hydroxy-5α-lanost-8-en-32-al	Mouse fibroblasts	371
24,25-Epoxy-5α-(24S)-lanost-8-en-3β-ol	IEC-6 Rat intestinal epithelial cells, ID_{50} ca.0.3 μM	370

Table 9. Inhibition of HMGCoA Reductase In Vitro by Oxysterols

Oxysterol	Cell System	Inhibition	Reference
Cholest-5-ene-3β,7α-diol *2*	Mouse fibroblasts	ID_{50} 2.5 μM	71, 355, 357, 372
Cholest-5-ene-3β,7β-diol *3*	Mouse fibroblasts	ID_{50} 1.9–2.7 μM	71, 355, 357, 372
3β-Hydroxycholest-5-en-7-one *4*	Human lymphoid cells	83% by 5 μg/mL	373
	Human lymphocytes	61% by 5 μg/mL; 98% by 25 μg/mL	153
	Human fibroblasts	5 μg/mL	374
	HeLa cells	58% by 1 μg/mL	336
	Glial cells	Inhibition	375
	Lung MRC-5 fibroblasts	ID_{50} 1.25 μg/mL	364
	Rabbit ileal mucosa	0.05–0.5 mM	367
	Chinese hamster ovary CHO-K1 cells	ID_{50} 1.5 μM	376
	Rat liver cells	ID_{50} 1.5 μM	377
	Rat embryo fibroblasts	93.5% by 6 μM	137
	Mouse fibroblasts	ID_{50} 1.7–2.5 μM	71, 355, 357, 372
(20S)-Cholest-5-ene-3β-20-diol (*10*)	Rat embryo fibroblasts	93.1% by 6 μM	137
	Mouse fibroblasts	ID_{50} 0.30–1.5 μM	71, 372
(22S)-Cholest-5-ene-3β,22-diol	Human lymphocytes	67% at 5 μg/mL; 95% at 25 μg/mL	141
(23R)-Cholest-5-ene-3β,23-diol	Human lymphocytes	35% by 5 μg/mL	141, 378
	Rat embryo fibroblasts	67% by 6 μM	137
(23S)-Cholest-5-ene-3β,23-diol	Human lymphocytes	89% by 5 μg/mL	141, 378
	Rat embryo fibroblasts	92.9% by 6 μM	137
(24R)-Cholest-5-ene-3β,24-diol	Human lymphocytes	85% at 5 μg/mL; 96% at 25 μg/mL	141
(24S)-Cholest-5-ene-3β,24-diol	Human lymphocytes	87% at 5 μg/mL; 97% at 25 μg/mL	141
Cholest-5-ene-3β,25-diol *7*	Human aorta smooth muscle cells	80% at 1 μg/mL	127, 147
	Human lymphocytes	93–95% at 5 μg/mL	141, 153
	Human keratinocytes	84% by 1.25 μM	365
	Human glioma cells	Inhibition	379
	Human fibroblasts	5 μg/mL	374, 380
	HeLa cells	78.5% by 1 μg/mL	336
	Vascular endothelium cells	90% at 2 μg/mL	191
	J774 macrophages	0.3 μg/mL	193
	Fibroblasts	0.1 μg/mL	193
	Dog ileal mucosa segments	100 μg/mL	384
	Chinese hamster ovary CHO cells	50% by 0.1 μg/mL	382
	Chinese hamster ovary CHO, TR-74 cells	Inhibition by 1 μg/mL	383
	Chinese hamster ovary CHO-K1 cells	80% at 0.25 μM	337, 384–386
	Chinese hamster ovary UT-1 cells	50% by ca.0.1 μg/mL	341
	Rat hepatocytes	50% by 50 μM	387, 388
	Rat C-6 glioma	2.5 μM	122
	Rat embryo fibroblasts	93.6% by 2.5 μg/mL	137, 149
	Rat embryo L_6 myoblasts	Inhibition by 0.16 μg/mL	145
	Rat IEC-6 intestinal epithelial cells	50% by ca.0.1 μg/mL	389
	Mouse fibroblasts	ID_{50} 0.05–0.17 μM	71, 372, 390, 391
	Mouse thymocytes	ID_{50} ca.4 μM	392
	Mouse P388D$_1$ macrophage-like cells	2.5 μM	393
	Chicken myoblasts	80% by 5 μg/mL	339, 340
(25R)-Cholest-5-ene-3β,26-diol *11*	Human lymphocytes	96% by 5 μg/mL	141
	Chinese hamster ovary CHO-K1 cells	36% at 0.25 μM	37, 386, 394
	Mouse fibroblasts	ID_{50} 0.26 μM	71
(25S)-Cholest-5-ene-3β,26-diol	Mouse fibroblasts	ID_{50} 0.16 μM	71
24,25-Epoxy-(24S)-cholest-5-en-3β-ol (*13*)	Mouse fibroblasts	ID_{50} ca.350 ng/mL	391, 395
3β-Hydroxy-5α-cholestan-6-one	Mouse fibroblasts	ID_{50} 0.8–1.5 μM	71, 372
5α-Cholestane-3,6-dione	Mouse fibroblasts	ID_{50} 0.8 μM	71
Cholesta-5,20(22)-diene-3β,25-diol	Rat embryo fibroblasts	93.8% at 6 μM	137
Cholesta-5,23-diene-3β,25-diol	Human lymphocytes	95% at 5 μg/mL	141
Cholesta-5,20(22)-diene-3β,25-diol	Human lymphocytes	68% at 5 μg/mL; 84% at 25 μg/mL	141
26-Norcholest-5-en-3β-ol	Human lymphocytes	89% at 5 μg/mL	141
3β-Hydroxycholest-5-en-22-one	Human lymphocytes	51% at 5 μg/mL; 96% at 25 μg/mL	141

Table 9. (Continued)

Oxysterol	Cell System	Inhibition	Reference
25-Fluoro-(22S)-cholest-5-ene-3β,22-diol	Human fibroblasts	>95% by 1 μg/mL	299, 361
	Cultured monkey smooth muscle cells	0.25 μg/mL	299
25-Methyl-(22S)-cholest-5-ene-3β,22-diol	Human fibroblasts	95% by 1 μg/mL	361
(22S)-26,27-Cyclocholest-5-ene-3β,22-diol	Human fibroblasts	>95% by 1 μg/mL	361
5α-Lanost-7-ene-3β,32-diol	Human lymphocytes	59% 11.25 μM	396
5α-Lanost-8-ene-3β,25-diol	IEC-6 Rat intestinal epithelial cells	ID_{50} 0.085 μM	370, 397, 398
24,25-Epoxy-5α-(24R)-lanost-8-en-3β-ol	IEC-6 Rat intestinal epithelial cells	ID_{50} ca.0.2 μM	370, 397, 398
24,25-Epoxy-5α-(24S)-lanost-8-en-3β-ol	IEC-6 Rat intestinal epithelial cells	ID_{50} ca.0.25 μM	370, 397, 398
25-Hydroxy-5α-lanost-8-en-3-one	IEC-6 Rat intestinal epithelial cells	ID_{50} 0.418 μM	370, 397, 398
(24ξ)-5α-Lanosta-8,25-diene-3β,24-diol	Human lymphocytes	93% at 5 μg/mL	141
(24ξ)-5α,9β-9,19-Cyclolanosta-8,25-diene-3β,24-diol	Human lymphocytes	99% at 5 μg/mL	141
(5Z,7E)-9,10-Secocholesta-5,7-diene-3β,25-diol	Human lymphocytes	63% at 5 μg/mL; 93% at 25 μg/mL	141
(5Z,7E,24R)-9,10-Secocholesta-5,7-diene-3β,24,25-triol	Human lymphocytes	66% at 5 μg/mL, 93% at 25 μg/mL	141
(5Z,7E)-9,10-Secocholesta-5,7-diene-1α,3β,25-triol	Human lymphocytes	20% at 5 μg/mL; 20% at 25 μg/mL	141

Table 10. Synthetic Oxysterols Inhibitory Actions in Mouse Fibroblasts (L-Cells)

Oxysterol	Sterol Biosynthesis, ID_{50}, μM	HMGCoA Reductase, ID_{50}, μM*	Reference
Cholest-5-ene-3β,19-diol	—	2.7	71
(22R)-Cholest-5-ene-3β,22-diol	—	8.2	71
(22S)-Cholest-5-ene-3β,22-diol	—	1.9	71
(24R)-Cholest-5-ene-3β,24-diol	—	0.63	395
(24S)-Cholest-5-ene-3β,24-diol	—	0.78	395
14α-Hydroxymethyl-5α-cholest-6-en-3β-ol	0.2	0.5–0.82	71, 348
14α-Hydroxymethyl-5α-cholest-7-en-3β-ol	2.0	3.3	348, 399
14α-Hydroxymethyl-5α-cholest-8-en-3β-ol	4.0	6.8	348
5α-Cholest-7-ene-3β,11α-diol	—	0.55	71
5α-Cholest-7-ene-3β,14α-diol	7.0	5.0	95, 400
5α-Cholest-7-ene-3β,15α-diol	—	0.50	71
5α-Cholestane-3β,15α-diol	—	0.50	71
(20S)-27-Norcholest-5-ene-3β,20-diol	—	0.77	71
27-Norcholest-5-ene-3β,25-diol	—	0.86	71
(20S)-26,27-Bisnorcholest-5-ene-3β,20-diol	—	1.2	71
(20S)-Chol-5-ene-3β,20-diol	—	9.7	71
5α,14β-Cholest-7-ene-3β,15α-diol	3.2	6.7	358
15β-Methyl-5α,14β-cholest-7-ene-3β,15α-diol	3.0	3.0	358
11α-Hydroxy-5α-cholest-7-en-3-one	—	0.50	71
14α-Hydroxy-5α-cholest-7-en-3-one	5.0	8.0	95, 400
14α-Hydroxymethyl-5α-cholest-6-en-3-one	0.8	1.0	348
14α-Hydroxymethyl-5α-cholest-7-en-3-one	4.0	2.0	348
15α-Hydroxy-5α-cholest-7-en-3-one	—	0.41	71
15α-Hydroxy-5α-cholestan-3-one	—	0.80	71
25-Hydroxycholest-4-en-3-one	—	3.5	71
3β-Hydroxy-5α-cholest-8-en-7-one	—	1.1	357
3β-Hydroxy-5α-cholest-8-en-11-one	—	9.0	357
3β-Hydroxy-5α-cholest-9(11)-en-12-one	—	1.0	71
3β-Hydroxy-5α-cholest-8(14)-en-15-one (*12*)	0.1	0.1–0.3	71, 95, 372
3β-Hydroxycholest-5-ene-22-one	—	1.4–3.2	71, 372
3β-Hydroxycholest-5-en-24-one	—	1.0	71
25-Hydroxycholesta-4,6-dien-3-one	—	1.8	71
25-Hydroxycholesta-3,5-dien-7-one	—	10	71
5α-Cholest-9(11)-ene-3,12-dione	—	1.0	71
5α-Cholest-8(14)-ene-3,15-dione	—	0.11	71
9α,11α-Epoxy-5α-cholest-7-en-3β-ol	—	2.3	357
14α,15α-Epoxy-5α-cholestan-3β-ol	5.0	4.0	95
24,25-Epoxy-(24R)-cholest-5-en-3β-ol (*13*)	—	1.57	395
24,25-Epoxy-(24S)-cholest-5-en-3β-ol	—	0.89	391, 395, 401

Table 10. (Continued)

Oxysterol	Sterol Biosynthesis, ID_{50}, μM	HMGCoA Reductase, ID_{50}, μM*	Reference
5α-Cholestane-3β,14α,15β-triol	4.8	—†	95
3β,25-Dihydroxycholest-5-en-7-one	—	0.48	71
3β,9α-Dihydroxy-5α-cholest-8(14)-en-15-one	—	0.40	71, 346
3β,14α-Dihydroxy-5α-cholestan-15-one	2.0	—†	95
14α-Hydroxy-5α-cholestane-3,15-dione	5.5	—†	95
3β-Hydroxy-5α-cholest-8(14)-en-15-one (*12*) oxime	0.7	0.2	95
5α-Cholest-8(14)-en-15-one	1.0	3.0	354
7α,15β-Dichloro-5α-cholest-8(14)-en-3β-ol	2	0.6	344
7α,15β-Dichloro-5α-cholest-8(14)-en-3β-ol 3β-benzoate	2	0.6	344
9α-Fluoro-3β-hydroxy-5α-cholest-8(14)-en-15-one	0.2	—	346
9α-Fluoro-5α-cholest-8(14)-ene-3,15-dione	0.2	0.20	71,346
4,4-Dimehtylcholest-5-ene-3β,7α-diol	—	1.5–1.7	71, 355, 357
4,4-Dimethylcholest-5-ene-3β,7β-diol	—	1.5–1.7	71, 355, 357
4,4-Dimethyl-5α-cholest-7-ene-3β,15α-diol	0.05	—	350
4,4-Dimethyl-5α-cholest-8-ene-3β,15β-diol	<1	—	350
3β-Hydroxy-4,4-dimethylcholest-5-en-7-one	—	1.5	71, 355, 357
3β-Hydroxy-4,4-dimethyl-5α-cholest-8(14)-en-15-one	0.05	—	350
14α-Hydroxymethyl-5α-cholest-7-ene-3β,15α-diol	0.2	0.4	348
14α-Hydroxymethyl-5α-cholest-6-ene-3β,15α-diol	0.7	0.5	348
15α-Hydroxy-14α-hydroxymethyl-5α-cholest-7-en-3-one	0.8	1.0	348
14α-Methyl-5α-cholestane-3β,7α,15α-triol	0.7	2.0	348
5α-Lanostane-3β,9α-diol	3.1	1.0	349
5α-Lanost-7-ene-3β,15α-diol	0.05	—	350
5α-Lanost-7-ene3β,15β-diol	<1	—	350
5α-Lanost-6-ene-3β,32-diol	—	0.44	71
5α-Lanost-7-ene-3β,32-diol	—	1.0–1.7	71, 372
5α-Lanost-8-ene-3β,32-diol	—	0.70–2.5	71, 371, 372
32-Methyl-5α-lanost-7-ene-3β,15α-diol	—	0.20	71
9α-Hydroxy-5α-lanostan-3-one	3.4	3.0	349
3β-Hydroxy-5α-lanost-7-en-15-one	<1	—	350
25,25-Epoxy-5α-(24R)-lanost-8-en-3β-ol	—	1.60	395
24,25-Eposy-5α-(24S)-lanost-8-en-3β-ol	—	0.68	395
7α,32-Epoxy-5α-4,4-bisnorlanostan-3β-ol	0.8	3.0	348
7α,32-Epoxy-5α-4,4-bisnorlanostane-3β,15α-diol	9	>74	348

*Comparison data for the 3β,7α-diol *2*, 2.5 μM; 3β,7β-diol *3*, 1.9–2.7 μM; 7-ketone *4*, 1.7 μM.[355,357]
†Not active at highest dose tested.[95]

data tend to support suppression of HMGCoA reductase activity as the primary site of action of oxysterols on de novo sterol biosynthesis, inhibitory actions occur at later steps of de novo sterol biosynthesis, including transformation of C_{30}-sterols to C_{27}-sterols[352] discussed later in this review and enzymes affecting C_{27}-sterol transformations.[351,362] It is crucially important to note that these actions of oxysterols on HMGCoA reductase are demonstrated only with intact mammalian cells. The isolated solubilized enzyme is partially degraded and unresponsive to oxysterols.

Several correlations between oxysterol structure and inhibitory actions may be made from these data. Although a 3β-hydroxyl group characterizes most, there is diminished activity in some but not all 3β-esters.[344,402] Moreover, a C-3 substituent is not necessary, 5α-cholest-8(14)-en-15-one (but not 5α-cholest-8(14)-ene-7α,15α-diol) being inhibitory. The 3α-chloro-5α-cholest-8(14)-en-15-one is also inactive.[71,354] Increased inhibitory activity is generally had for oxysterols with a greater spacial separation between the C-3 and second oxygen function, thus with oxygen function in the D-ring or side-chain.[322,323,403] However, the means by which oxysterol be presented cultured cells,[404,405] differential uptake of oxysterol by cultured cells,[328] varying cell densities during bioassay,[406] and metabolic deactivation may influence bioassay results.

The suppression of de novo sterol biosynthesis in most of these cases appears to be via inhibition of HMGCoA reductase, thus by the now anticipated mode of action of the natural oxysterols. However, bioassay data in L cells (Table 10) for four of these oxysterols indicate that suppression of HMGCoA reductase activity is not the mode by which de novo sterol biosynthesis is inhibited. Whether these synthetic oxysterols affect these results by insertion into the L cells plasma membrane, by inhibitions at later phases of de novo sterol biosynthesis (oxidative removal of the 4- and 14-

methyl groups), or by some as yet undiscovered process remains uncertain.

In a few instances synthetic oxysterols have been examined in vivo, but development of an effective and safe oxysterol pharmacological agent for control of plasma cholesterol levels has yet to materialize.[407] One of the most promising is the 15-ketone *12* which effectively inhibits HMGCoA reductase activity and de novo sterol biosynthesis in vitro and displays an in vivo hypocholesterolemic action in rats, rhesus monkeys, and baboons[53,95,353,408-411] but also may reduce intestinal absorption of dietary cholesterol[412] and provoke toxic effects of appetite and weight loss in rats fed the oxysterol.[95]

Indeed, the 15-ketone *12* has a potential advantage of being metabolized to cholesterol both in vitro[413] and in vivo,[414,415] a metabolic fate also shared by another de novo sterol biosynthesis inhibitor 5α,14β-cholest-7-ene-3β,15α-diol.[416]

The 15-ketone *12* has other actions as well; cytosol acetoacetyl coenzyme A thiolase and HMGCoA synthase are inhibited in cultured cells in the same manner as HMGCoA reductase.[334] Furthermore, esterification of cholesterol by rat liver and jejunum microsomal ACAT in vitro is inhibited at the same time the 15-ketone *12* is esterified. Jejunum ACAT is more sensitive to inhibition by *12* than liver ACAT.[417] The inhibition is also achieved in vivo.[418] These results may account for the diminished intestinal absorption of dietary cholesterol on rats fed 0.05% 15-ketone *12*.[412] The stimulated action of jejunum ACAT on 15-ketone *12* also provides basis for the metabolism of 15-ketone *12* to fatty acyl esters in animals fed the oxysterol.[414,415]

Of side-chain variant oxysterols 25-fluoro-(22S)-cholest-5-ene-3β,22-diol and 25-methyl-(22S)-cholest-5-ene-3β,22-diol suppress HMGCoA reductase activity in vitro in human fibroblasts and in vivo in liver of rats fed 0.5 mg/kg. Total plasma cholesterol levels are lowered by 15–18% in rhesus monkeys fed either oxysterol at 10 mg/kg/day.[299,361] Liver homogenates from mice fed the degraded cholesterol autoxidation product pregn-5-en-3β-ol (0.25–0.50% in the diet) also exhibited diminished de novo sterol biosynthesis.[94]

Not all oxidized sterol inhibitors of de novo sterol biosynthesis need be oxygenated, as cholesta-5,7,9-trien-3β-ol but not cholesta-5,7-dien-3β-ol nor 5α-cholesta-7,9-dien-3β-ol is inhibitory.[364] Also, other unoxidized cholesterol derivatives may suppress HMGCoA reductase and de novo sterol biosynthesis; water soluble cholesterol polyoxyethyl ethers (10 μM) inhibit HMGCoA reductase and de novo sterol biosynthesis in cultured human skin fibroblasts but also interfere with de novo fatty acid biosynthesis, thus involving different modes of action than oxysterols which do not affect fatty acid metabolism.[419] An opposite effect on de novo sterol biosynthesis is obtained in rats fed 2% cholesterol polyoxyethyl ethers, where increased hepatic de novo sterol biosynthesis results from reduced intestinal absorption of dietary cholesterol.[420] An analogue cholesterol methoxypolyoxyethyl ether inhibits HMGCoA reductase of cultured rat liver cells (ID_{50} 2.5 μM); in this case the inhibition appears to be via decrease in amount of reductase, and a decline in HMGCoA reductase messenger RNA preceeds decline in HMGCoA reductase.[377]

Water soluble cholesterol 3β-sulfate also suppresses HMGCoA reductase activity in cultured human fibroblasts.[380] As oxysterols, cholesterol 3β-sulfate, and cholesterol (LDL) all suppress HMGCoA reductase activity and thereby de novo sterol biosynthesis in vitro, attention is drawn to the question of how these effects are accomplished. In that all three are present in vivo in the human and oxysterols and cholesterol are present in the human diet, there is also raised the question whether any one be the sole agent implicated in vivo or whether oxysterol, cholesterol, and cholesterol 3β-sulfate interact in unprobed complex fashion in physiological reality.

These several items implicating oxysterols in the suppression of HMGCoA reductase activity and thereby of de novo sterol biosynthesis provide the basis for an hypothesis that endogenous oxysterols regulate HMGCoA reductase activity in vivo. The hypothesis initially put forth by Chen and Kandutsch but since modified to take into account more recent discoveries of inhibitory oxidized lanosterol derivatives[421] suggests that C_{27}-oxysterols such as the 3β-25-diol *7* formed from cholesterol and desmosterol 24,25-epoxide (*13*) derived from (3S,22S)-squalene 2,3;22,23-bisepoxide serve as endogenous regulators of HMGCoA reductase activity through initial interaction with an intracellular protein, the "oxysterol binding protein", followed by action of the protein-bound oxysterol at or in the cell nucleus to suppress de novo synthesis of HMGCoA reductase.[422,423] The hypothesis is outlined in Figure 2.

The putative regulatory system is likened to those implicated in the actions of mammalian C_{18}-, C_{19}-, and C_{21}-steroid hormones where similar interactions with intracellular proteins (receptors) and the nucleus provoke de novo messenger RNA biosynthesis and subsequent protein biosynthesis. However, fundamental differences between the mechanisms by which mammalian steroid hormones exert their actions and the oxysterol inhibition of de novo sterol biosynthesis have been noted.[392] These matters remain highly speculative but offer stimulating opportunities for further examination of the mechanisms by which oxysterols affect

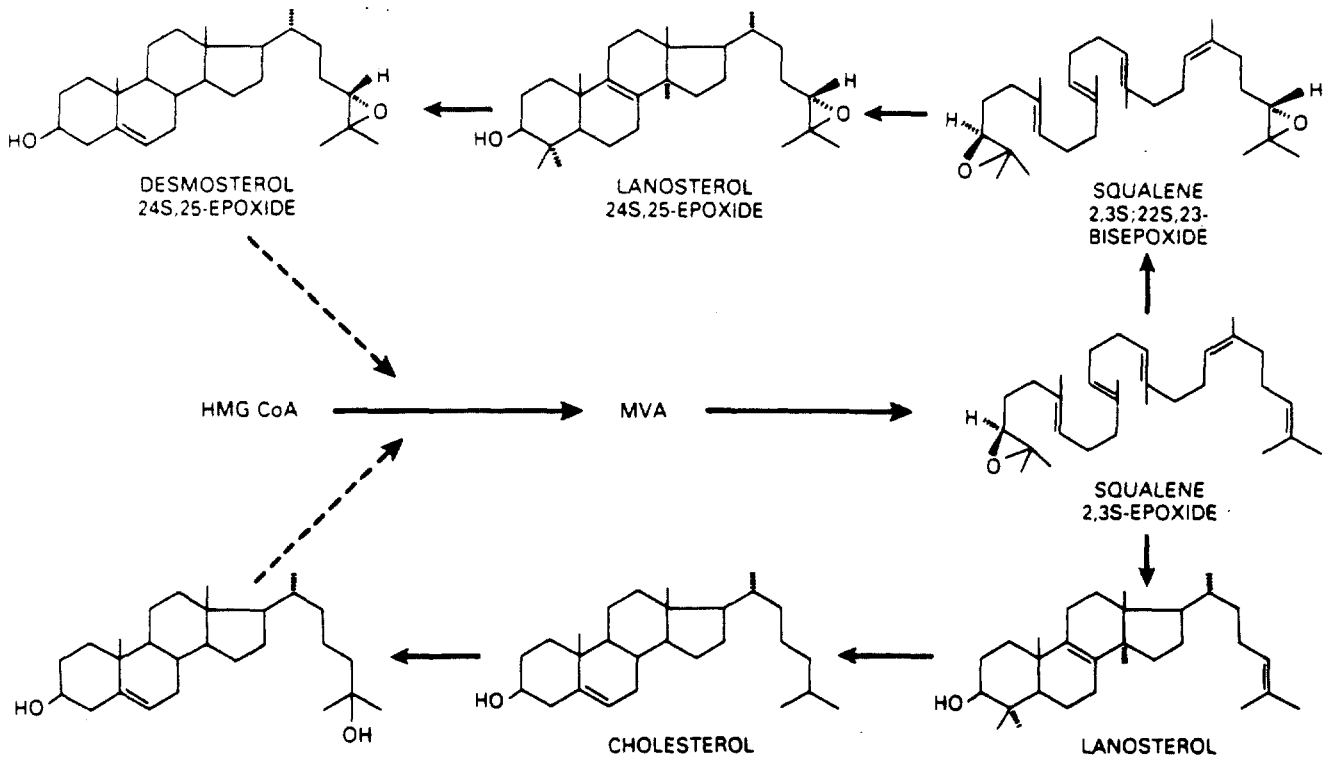

Fig. 2. Steps implicated in the oxysterol regulatory hypothesis. Abbreviations: HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonate.

cellular processes and for understanding the physiological significance of the presence of endogenous oxysterols in vivo.

The oxysterol regulatory hypothesis poses that the selected putative regulatory oxysterols 3β,25-diol *7* and 24,25-epoxide *13* be formed within the target cell, thus as a local hormone, but the hypothesis may also include plasma oxysterols as possible agents, thus as true hormones. As plasma levels of oxysterols exceed those suggested as regulatory, if plasma oxysterols be implicated, necessarily an effective, selective means of limiting entry of such oxysterols into the cell or of diverting oxysterols from influencing cellular processes must also exist.

The oxysterols implicated thus may have divers origins, including dietary origins in which plasma transport be involved but also metabolic origins via endogenous de novo sterol biosynthesis[424,425] and by actions of cytochrome P450 mixed function oxidases catalyzing sterol hydroxylation and oxidative scission of the 4- and 14-methyl groups of C_{30}-sterols.[330,331,398,426-429] Also implicated is an alternative de novo sterol biosynthesis pathway in which (3S,22S)-squalene 2,3;22,23-bisepoxide derived from (3S)-squalene 2,3-epoxide[330,331,389,397,429] cyclizes to the lanosterol epoxide 24,25-epoxy-5α-(24S)-lanost-8-en-3β-ol, from which the desmosterol epoxide *13* is formed.[395,430,431] These processes thus implicate the 3β,25-diol *7* and the 24,25-epoxide *13*[432-436] (detected in human liver[395,401]) and the 32-aldehydes 3β-hydroxy-5α-lanost-8-en-32-al[396,437,438] and 3β-hydroxy-5α-lanosta-8,24-dien-32-al[371] (lanosterol metabolies and cholesterol precursors) as regulatory oxysterols.

The 3β,25-diol *7* has attracted much attention as an exogenous oxysterol HMGCoA reductase inhibitor but also as a putative endogenous oxysterol regulator of de novo sterol biosynthesis. The ready commercial availability and high potency of the 3β,25-diol *7* appears to motivate much of the work directed at these aspects, but physical properties of the 3β,25-diol *7* in water may be important. The 3β,25-diol *7* is more water soluble than cholesterol, and perhaps more significantly is unassociated in water (critical micellar concentration 0.62–3.10 μM), thus in distinction to cholesterol where micellar associations occur (critical micellar concentration 13.0–64.8 μM).[404] These properties suggest that the 3β,25-diol *7* may be more accessible to interactions with proteins and membranes than is cholesterol. However, the physical form in which exogenous oxysterol be presented as exogenous agents to cultured cells may be quite varied, with unknown specific fates.[405]

Similar measurements on other endogenous oxysterols have not been reported, but one may project

that the same properties exist. Moreover, other endogenous oxysterols such as the 3β,26-diol *11* long recognized as implicated in human atherosclerosis, being less available, have not been so fully studied. Nonetheless, in cultured Chinese hamster ovary CHO-K1 cells the 3β,26-diol *11* exceeded the inhibitory potency of the 3β,25-diol *7*, inhibiting 36% versus 20% at 0.25 μM.[37,386,394] An ad hoc argument that the 3β,26-diol *11* cannot account for the inhibitory actions of plasma LDL in vitro has been made, despite high levels of the 3β,26-diol *11* in human plasma and its high inhibitory potency.[439] The argument ignores the possible contribution of plasma oxysterols absorbed by mechanisms other than the LDL receptor-mediated uptake.

A bioassay-directed search of plasma and cultured cells for inhibitory oxysterols discovered the 3β,25-diol *7* and 24,25-epoxide *13* as the active oxysterols in cultured cells.[371,391,440] This close association of 3β,25-diol *7* with 24,25-epoxide *13* has led to the additional speculation that the 3β,25-diol *7* be a metabolite of 24,25-epoxide.*13* However, this transformation has not been demonstrated, and liver metabolism of isomeric 24,25-epoxides 24,25-epoxy-5α-(24R)-lanost-8-en-3β-ol and 24,25-epoxy-(24R)-cholest-5-en-3β-ol yields the corresponding 3β,24-diols 5α-(24R)-lanost-8-ene-3β,24-diol and (24R)-cholest-5-ene-3β,24-diol.[395,441] Moreover, the 3β,25-diol *7* has an established origin in the enzymic 25-hydroxylation of cholesterol and is also a cholesterol autoxidation product present in cholesterol-rich processed foods. The 24,25-epoxide *13* as an end-product of the alternative de novo sterol biosynthesis pathway from (3S,22S)-squalene 2,3;22,23-bisepoxide is also an autoxidation product of desmosterol.[442]

A key feature of the regulatory hypothesis is that cytosol proteins bind the regulatory oxysterol (but not cholesterol) with high affinity for ultimate biological action or disposal. Proteins binding oxysterols occur in sheep endocrine tissues (binding the 3β,25-diol *7*, 3β,20-diol *10*, (20R,22S)-cholest-5-ene-3β,22-diol, and (20R,22R)-cholest-5-ene-3β,22-diol)[443,444] and rat liver (binding the 3β,7-diols *2* and *3* and 7-ketone *4*),[445] but these proteins also bind cholesterol more effectively.

However, cytosol proteins have been discovered in mouse liver and spleen, in rat liver, and in several established cultured cell lines that exhibit high affinities for oxysterols that are correlated with the potency of the oxysterol in suppressing de novo sterol biosynthesis and HMGCoA reductase activity in vitro.[71,372,376,446–449] Similar oxysterol binding proteins occur in human lymphocytes and rat embryo fibroblasts; however, in these instances the protein recognizes oxysterols that are active in suppressing de novo DNA biosynthesis. Oxysterols such as the 7-ketone *4* and (23R)-cholest-5-ene-3β,23-diol that suppress HMGCoA reductase activity but not de novo DNA biosynthesis are but poor competitors for the oxysterol binding protein in comparison with the 3β,25-diol *7*.[137,153,378]

These oxysterol binding proteins are different from the recognized intracellular sterol binding proteins and receptor proteins for C_{18}-, C_{19}-, and C_{21}-steroid hormones[378,447] and exhibit high affinity for the active oxysterols but not for cholesterol. The binding is saturable, reversible, and subject to competition among the active oxysterols. The native protein from mouse fibroblast L cells appears to be at least a trimeric species (sedimentation coefficient variously 7.5–8S, M_r 236,000) which binds oxysterol and looses a subunit, yielding a oxysterol-dimeric protein complex (7.5S, M_r 169,000) that may be further dissociated irreversibly to a 4.2S subunit of M_r 97,000.[402,450–454]

Nonspecific oxysterol binding occurred in some of these cases, and oxysterols may be bound to other cytosol proteins. For example, the 7-ketone *4* binds to an antiestrogen binding protein of chicken liver.[455] The significance of such intracellular protein binding remains uncertain, but the more specific binding in cultured cells has led to the viewpoint that the binding proteins are oxysterol receptors implicated in endogenous regulation of de novo sterol biosynthesis by oxysterols.[452,454] Intracellular levels of sterol carrier protein implicated in cholesterol biosynthesis and metabolism also appear to be increased in cultured human fibroblasts by the 3β,25-diol *7*.[456]

On another note, reversible phosphorylation accords rapid modulation of HMGCoA reductase activity, the phosphorylated enzyme being deactivated. As many as three different phosphorylation systems are implicated: a bicyclic cascade involving reductase kinase and reductase kinase kinase, protein kinase C, and a Ca^{2+}/calmodulin-dependent reductase kinase.[315–317,332,457,458] Oxysterols are implicated in the regulatory actions of the phosphorylation cascade, in the inhibition of calmodulin, and in effects on membranes affecting cellular Ca^{2+} influx and efflux (previously treated in the Membrane Effects section of this review). Each of these events accords rapid modulation of HMGCoA reductase activity.

The deactivation of HMGCoA reductase by phosphorylation appears to be correlated with activation by phosphorylation of enzymes important to cholesterol metabolism, thus of ACAT and cholesterol 7α-hydroxylase.[459] Phosphorylation and dephosphorylation

of the three enzymes appears to provide a basis for rapid adjustments in maintenance of cholesterol homeostasis.

However, it be nuclear events putatively regulated by oxysterol that have captured so much attention, and much evidence implicates the cell nucleus in oxysterol inhibition of HMGCoA reductase activity. The oxysterol actions occur only in intact cells and not in enucleated cells.[460] Oxysterol-resistant mutant cultured cells have been isolated, it being evident that oxysterol resistance be a dominant selectable genetic trait.[333,337,338,382,461-464] Intracellular proteins are implicated in oxysterol modulation of de novo sterol biosynthesis,[456,465] and de novo protein biosynthesis is involved in the regulation of cellular HMGCoA reductase activity by oxysterols, both for enzyme synthesis and degradation.[145,337-341,377,383-385,466-470]

Oxysterols are not bound by nuclear chromatin nor do they interfere with the binding of cholesterol,[471] but it is postulated that the oxysterol-binding protein complex be the reactive species binding in the nucleus. At the nucleus oxysterols appear to diminish the amount of messenger RNA for HMGCoA reductase, thus to act by transcriptional control.[377,469,472,473] Nonetheless, translational control may also occur in some circumstances.[339,340,463,464]

Cholesterol homeostasis appears to be influenced by several coordinated actions of oxysterols, including suppressed HMGCoA reductase and LDL receptor activities and concommitant increased degradation of HMGCoA reductase accompanied by inhibition of HMGCoA synthase activity.[338-341,377,382,383,470] In that the intracellular effects of LDL, cholesterol, and oxysterols appear to be common ones in these several systems, common controlling factors have been posed.[325,333] However, the broad oxysterol structure-activity relationships, implications of membrane phenomena, protein phosphorylation, and Ca^{2+} metabolism as well as of nuclear events and matters dealing with dietary and plasma oxysterols in vivo leaves the topic open for further exploration.

There now appear to be at least four different mechanisms by which HMGCoA reductase activity be modulated in vitro: 1) by membrane phenomena, including possibly altered membrane fluidity but also including altered Ca^{2+} fluxes affecting enzymic phosphorylation; 2) reductase phosphorylation by Ca^{2+}/calmodulin-dependent and reductase kinase actions influenced by events stimulated by oxysterol; 3) de novo biosynthesis involving nuclear events (protein biosynthesis); and 4) reductase degradation, also involving protein biosynthesis.

The relative contributions of these factors in vivo are totally unknown, but it appears that oxysterols, whether of dietary or endogenous sources, may be implicated.

Acyl-coenzyme A: Cholesterol O-acyltransferase (EC 2.3.1.26)

Intracellular microsomal acyl-Coenzyme A: cholesterol O-acyltransferase (ACAT) affecting cholesterol esterification is coordinately regulated with HMGCoA reductase, the two enzymes having diurnal rhythms effectively twelve hours out of phase with one another.[474] Together the two enzymes control de novo sterol biosynthesis and sterol metabolism, and oxysterols affect both processes, although by different mechanisms.

Oxysterol effects on ACAT are summarized in Table 11. The stimulatory actions occur in cultured cells and in isolated microsomes and appear to result from an increase in sterol substrate availability rather than by a direct action on the enzyme.[393,486,491,493,494] Nonetheless, the effect has been suggested to be one of increasing ACAT activity.[480] However, oxysterols do not affect changes in ACAT properties K_m and V_{max}.[495] The increased substrate availability may be from an increased uptake of exogenous cholesterol into cultured cells.[475]

In cultured hepatocytes as the amount of cholesterol ester increases under oxysterol stimulation, the amount of cholesterol ester in VLDL secreted also is increased.[483]

However, ACAT activity is sensitive to the immediate lipid environment within the endoplasmic reticulum, both stimulatory and inhibitory effects occurring depending on membrane lipid composition. Increased lipid peroxidation of the microsomes does not appear to be a significant factor.[496] Improving substrate availability by use of aqueous cholesterol dispersions in detergent increases ACAT activity,[497] and decreasing substrate availability by prior transformation of microsomal cholesterol to the 3-ketone *9* by cholesterol oxidase diminishes ACAT activity.[498] However, it is uncertain whether oxysterols formed in the aqueous dispersions by cholesterol autoxidation or effects of the 3-ketone *9* formed by cholesterol oxidase influenced results.

The in vitro oxysterol stimulation of ACAT in rat liver microsomes does not occur in microsomes from bovine adrenal cortex[499,500] nor from murine macrophage-like cells,[393] it being reasoned that ACAT in these cases be already saturated with sterol substrate so that oxysterol stimulation not occur. The specific oxysterol 3β,25-diol *7* does not have an effect on in-

Table 11. Oxysterol Effects on Acyl Coenzyme A: Cholesterol O-Acyltransferase (EC 2.3.1.26)

Oxysterol	Bioassay System	Effect on Sterol Esterification	Reference
3β-Hydroxycholest-en-7-one *4*	Human FH fibroblasts GM 1915	Stimulation by 12.5 μM	475
	Cultured rat hepatocytes	Stimulation by 10 μg/mL	388
(22S)-Cholest-5-ene-3β,22-diol	Human skin fibroblasts, monkey artery smooth muscle cells	Inhibition	299, 361, 369
Cholest-5-ene-3β,25-diol *7*	Human monocyte-derived macrophages	Stimulation by 10 μg/mL	476
	Preconfluent human keratinocytes	Stimulation (4.8-fold) by 2.5 μM	119
	Human CaCo-2 enterocytes	Stimulation	477
	Bovine vascular endothelium	Stimulation (3- to 4-fold) by 2 μg/mL	191
	Rabbit jejunum explants	Stimulation	478
	Rabbit enterocytes	Stimulation (5-fold)	479–481
	Rabbit intestinal microsomes	Stimulation by 8–10 μg/mL	480, 481
	Chinese hamster ovary cells	Stimulation	482
	Cultured rat hepatocytes	Stimulation (3- to 6-fold) by 10 μg/mL	388, 483
	Rat intestinal epithelium IEC-6 cells	Stimulation by 2 μM	370
	Intestinal explants	Stimulation	478
	Rat hepatic microsomes in vitro	Stimulation by 26–100 μM	484–486
	Murine macrophage-like cells	Stimulation	393, 487
	BALB/c Mouse fibroblasts	Stimulation by 5 μg/mL	488
	Mouse P388D$_1$ macrophage-like cells	Stimulation	393
	Mouse peritoneal macrophages	Stimulation by 2–20 μg/mL	156
25-Fluoro-(22S)-cholest-5-ene-3β,22-diol	Human skin fibroblasts, monkey artery smooth muscle cells	Inhibition	299, 361
Pregn-5-en-3β-ol	Rat liver microsomes	Inhibition	489
Androst-5-en-3β-ol	Rat liver microsomes	Inhibition	490
3β-Hydroxy-17β-isohexyloxyandrost-5-en-7-one (SC31769)	Microsomes in vitro	Inhibition	484
5α-Lanost-8-ene-3β,15α-diol	Rat liver S_{10} homogenate	Stimulation	491
5α-Lanost-8-ene-3β,15β-diol	Rat liver S_{10} homogenate	Stimulation by 54 μM	491
5α-Lanost-8-ene-3β,25-diol	Rat intestinal epithelium IEC-6 cells	Stimulation by 2 μM	370
24,25-Epoxy-5α-(24R)-lanost-8-en-3β-ol	Rat intestinal epithelium IEC-6 cells	Stimulation by 2 μM	370
24,25-Epoxy-5α-(24S)-lanost-8-en-3β-ol	Rat intestinal epithelium IEC-6 cells	Stimulation by 2 μM	370

testinal mucosal cell cytosolic cholesterol esterase that also esterifies cholesterol intracellularly.[481]

Moreover, some oxysterols inhibit ACAT activity. Progesterone inhibits the enzyme, apparently by binding to the enzyme at a site remote from the active site,[485,486,492] (22S)-Cholest-5-ene-3β,22-diol and other synthetic oxysterols also inhibit ACAT.[299,361,484] The (22S)-3β,22-diol concommitantly reduced de novo sterol biosynthesis, inhibited sterol esterification, and diminished the number of plasma membrane LDL receptors in cultured cells.[299,361] The 15-ketone *12* that effectively inhibits HMGCoA reductase also inhibits liver and jejunum microsomal ACAT in vitro and in vivo![417,418]

Stimulatory 3β,25-diol *7* is itself also esterified,[486,495] but there is some question whether the enzyme implicated be ACAT.[489,495,501] A similar case obtains for 5α-lanost-8-ene-3β,15β-diol which is esterified at both hydroxyl groups by liver enzymes and which stimulates sterol esterification.[491] The 15-ketone *12* is itself esterified in vitro[70,417] and in vivo in a baboon fed the oxysterol.[415]

These results have not lead to a unified viewpoint on how oxysterols affect ACAT.

Other enzymes of sterol metabolism

Although the effects of oxysterols on HMGCoA reductase and ACAT have received the bulk of study on such matters, other specific enzymes implicated in sterol metabolism and in other cellular functions also are influenced by oxysterols. Among enzymes of sterol metabolism are 3β-hydroxysteroid dehydrogenase, cholesterol 5,6-epoxide hydrolase, cholesterol 7α-hy-

droxylase, the cholesterol side-chain cleavage system, and methylsterol oxidases. Other enzymes affected are placental C_{19}-steroid aromatase, 5'-nucleotidase, and Na^+/K^+ ATPase systems already discussed.

3β-hydroxysteroid dehydrogenase (EC 1.1.1.145). The enzyme is inhibited 51% in adrenal microsomes from male rats fed the 3-ketone *9*.[502] However, neither cholesterol nor the 3β,20-diol *10* are good substrates for the adrenal cortex enzyme,[503] so this in vivo inhibition is difficult to interpret.

Cholesterol 5,6-epoxide hydrolase (EC 3.3.2.3). A microsomal cholesterol 5,6-epoxide hydrolase different from other epoxide hydrolases present in lung and liver is implicated in transforming either isomeric 5,6-epoxide *5* or *8* with equal facility to the 3β,5α,6β-triol *6*.[504,505] Both 5,6-epoxides and triol are toxic agents in many bioassays, and the hydrolase creating triol from either epoxide is subject to inhibitions by oxysterols.

Rat liver epoxide hydrolase is sensitive to product 3β,5α,6β-triol *6* inhibition and is inhibited by the 7-ketone *4*, 3β-hydroxy-5α-cholestan-6-one, and 3β-hydroxy-5α-cholestan-7-one. Either 5,6-epoxide *5* or *8* also inhibits the hydrolysis of the other.[47,244,245,505,506] Besides inhibition by the 3β,5α,6β-triol *6* the isomeric triols 5α-cholestane-3β,5,6α-triol, 5β-cholestane-3β,5,6α-triol, and 5β-cholestane-3β,5,6β-triol also inhibit the hydrolase.[506]

The synthetic oxysterols 5,6α-epoxy-5α-cholest-7-en-3β-ol and 5,6β-epoxy-5β-cholest-7-en-3β-ol are potent inhibitors of cholesterol 5,6-epoxide hydrolase, apparently acting by covalently binding with the enzyme.[88] The ID_{50} 0.048 μM for the 5β,6β-epoxide isomer is lower than that of 5,6α-imino-5α-cholestan-3β-ol (0.085 μM),[506] the best inhibitor heretofore.[507]

Cholesterol 7α-hydroxylase (EC 1.14.13.17). Microsomal cholesterol 7α-hydroxylase recognized as a regulated initial step in hepatic cholic acid biosynthesis is subject to oxysterols inhibition in vitro. The enzyme appears to be the cytochrome $P450_{LM4}$ induced in rabbit liver by cholestyramine.[511] Among the oxysterol inhibitors are the enzyme product 3β,7α-diol *2*, the epimeric 3β,7β-diol *3*, 7-ketone *4*, and 3β-hydroxychol-5-enic acid, all formed in vitro and potentially in vivo, these inhibiting the enzyme competitively.[508,509]

Product inhibition by 3β,7α-diol *2* during bioassay may not be a limitation,[509] and as elevated levels of 3β, 7α-diol *2* in vivo appear to parallel levels of 7α-hydroxylase activity,[25] the inhibition may not be significant in vivo. However, 3β,7β-diol *3* also formed via lipid peroxidation of cholesterol in vivo is a more effective (competitive) inhibitor (K_i 2.4 μM compared to K_m 140 μM for substrate cholesterol).[509]

Oxysterols may also inhibit later steps in the biosynthesis of bile acids; for example, the 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one or 5α-cholestane-3β,7α-diol implicated in cholic acid biosynthesis is also catalyzed by a microsomal cytochrome P450 enzyme subject to oxysterol competitive inhibitions in vitro.[510] Effective oxysterol inhibitors included 5α-cholestane-3β,7α-diol, 5α-cholestane-3β,7α,25-triol, 7α-hydroxycholest-4-en-3-one, and 7α,25-dihydroxycholest-4-en-3-one, only 7α-hydroxycholest-4-en-3-one being formally a cholesterol autoxidation product. However, the 7-ketone *4* and 3β,25-diol *7* inhibit later steps in cholic and chenodeoxycholic acids biosynthesis by isolated rat hepatocytes.[511]

Cholesterol side-chain cleavage system (EC 1.14.15.6). Scission of the isohexyl moiety of the sterol side-chain by endocrine tissues yields 3β-hydroxypregn-5-en-20-one (pregnenolone) required by steroid hormone biosyntheses. Pregnenolone may then be transformed to corticosteroids, progesterone, androgens, etc. depending on tissue. The scission process involves initial substrate binding to mitochondrial cytochrome $P450_{scc}$ with subsequent $22\beta_F$-hydroxylation followed by $20\alpha_F$-hydroxylation to (20R,22R)-cholest-5-ene-3β,20,22-triol and carbon-carbon bond cleavage yielding pregnenclone.

Binding of sterol substrate to the enzyme (evinced by characteristic changes in absorption spectra) is crucial to the process. Oxysterols cholest-5-ene-3β,4β-diol, 3β,7α-diol *2*, 3β,7β-diol *3*, 3β,20-diol *10*, (22R)-cholest-5-ene-3β,22-diol, and 3β,25-diol *7* all bind to adrenal cytochrome $P450_{scc}$, and all appear to be substrates for metabolism.[279,512-517] The oxysterol 3β-hydroxycholest-5-en-22-one also binds to cytochrome $P450_{scc}$ but cannot be a substrate, thus is a competitive inhibitor of the enzyme.[518]

The 3β,25-diol *7* is a good substrate for side-chain cleavage[519,520] and in its study there arises an apparent paradox. The 3β,25-diol *7* inhibits pregnenolone formation from exogenous (labeled) cholesterol in rat adrenal mitochondria in vitro[517] but stimulates production from endogenous precursors in human fetal adrenal mitochondria.[519] In the first instance the 3β,25-diol *7* appears to be in competition with labeled substrate, thereby decreasing labeled product formed, whereas in the second case the 3β,25-diol *7* served as substrate together with endogenous sterol to increase pregnenolone levels formed. The issue is thus merely a matter of different assays employed. A similar case obtains for the 3β,20-diol *10* with adrenal cortex, corpus luteum, and placenta $P450_{scc}$ where labeled cholesterol

substrate must compete with 3β,20-diol *10* also a substrate.[520-522]

Other oxidative side-chain cleavages are influenced by oxysterols. The diminution of adrenocorticotropin-stimulated corticosterone levels in cultured rat adrenal cells by (22S)-cholest-5-ene-3β,22-diol, 3β-hydroxy-cholest-5-en-22-one, and (20S)-cholest-5-ene-3β,17α,20-triol may also occur by oxysterol inhibition of the side-chain cleavage system, probably at the initial sterol-enzyme binding interaction.[524,525] By contrast, the luteinizing hormone-stimulated increase in testosterone production in cultured murine testis Leydig cells is further increased by (22R)-cholest-5-ene-3β,22-diol,[526] and some rat Leydig cell cultures are also stimulated by the 3β,25-diol *7*.[526,527] Similar cases exist in the gonadotropin-stimulated biosynthesis of progesterone in cultured rat ovary luteal cells[531,532] and in the biosynthesis of 20-hydroxy-(20S)-pregn-4-en-3-one in cultured rat ovary granulosa cells where exogenous 3β,25-diol *7* stimulates an increase in products.[530]

It is uncertain whether the observed oxysterol stimulations of C_{21}-steroid biosynthesis result from mere increased substrate levels represented by the exogenous oxysterol or from an increase in enzyme activity.

The recent demonstration of cytochrome $P450_{scc}$ in rat brain now provides a basis for the accumulation of pregnenolone therein.[531,532] This discovery taken with our past demonstration of (24S)-cholest-5-ene-3β,24-diol accumulation in developing rat brain[43] and inhibition of cytochrome $P450_{scc}$[1] now offers speculatively another potential reason for the presence of (24S)-cholest-5-ene-3β,24-diol in brain, namely modulation of cytochrome $P450_{scc}$ as well as HMGCoA reductase, etc.

Methylsterol oxidases. Although the de novo biosynthesis of cholesterol is controlled by HMGCoA reductase in turn inhibited by oxysterols, inhibition at later stages in the biosynthesis process by oxysterols is also indicated, particularly at those steps involved in the transformation of lanosterol to cholesterol. For instance, methylsterols accumulate as products in guinea pig lymphocytes cultures treated with the 3β,7β-diol *3* or 3β,25-diol *7*.[362]

Oxysterols appear to limit the in vitro enzymic conversion of lanosterol or 5α-lanost-8-en-3β-ol (dihydrolanosterol) to cholesterol by inhibition of the mixed function oxidase removal of one or more of the 4- and 14α-methyl features. The inhibition of a liver microsomal 4-methylsterol oxidase by 50 μM 3β,5α,6β-triol *6* or 3β,25-diol *7* has been reported.[533] Also, the specific inhibition of the 4-methylsterol oxidase by synthetic analogs 4-allenyl-5α-cholestan-3β-ol (K_i 54 μM), 4α-cyanoethynyl-5α-cholestan-3β-ol (K_i 45 μM), and 4α-oxiranyl-5α-cholestan-3β-ol (K_i 107 μM) is described.[534]

Furthermore, the 14α-methylsterol oxidase system is inhibited by lanostane derivatives modified at the 14α-methyl feature, including 32-methylene-5α-lanost-7-en-3β-ol, 14-ethynyl-4,4-dimethyl-5α-cholest-7-en-3β-ol, 32,32-difluoro-5α-lanost-7-en-3β-ol, 32-difluoromethyl-5α-lanost-7-en-3β-ol, 32-ethynyl-5α-lanost-7-en-3β-ol, 32-ethynyl-5α-lanost-7-ene-3β,32-diol, and 5α-lanost-7-ene-3β,32-diol.[535]

As with oxysterols synthesis programs seeking effective chemotherapeutic agents against tumors and for lowering plasma cholesterol via inhibition of de novo sterol biosynthesis, so also synthesis programs for oxysterols inhibiting the transformation of C_{30}-sterols to cholesterol have been devised.[536-538] In these matters, the oxidative removal of the 14-methyl group is suspected as the step inhibited. The data of Table 12 establish that the oxidation of C_{30}-sterols to cholesterol is indeed subject to inhibitions by oxysterols.

It may be seen that several 15α-hydroxysterols and 3β-hydroxy-5α-lanost-8-en-7-one are very effective inhibitors of lanosterol metabolism to cholesterol, while other oxysterols are inactive. 3β-Hydroxy-5α-lanost-8-en-7-one is also effective in lowering plasma cholesterol in vivo when fed rats at a level of 0.1% in the diet.[96]

CONCLUSIONS

The items reviewed here evince the capacity of oxidized sterols to affect important metabolic processes and provide a basis for recognition of the potential significance of the presence of oxysterols in living systems. However, a definitive or even satisfying viewpoint integrating these many oxysterols effects with human health interests has yet to emerge from these accounts. As the oxidation of cholesterol in biological and chemical systems depends on the properties of the system studied, witness the presence of some cholesterol autoxidation products but not others in tissues and foods, so also the question of biological activities and more importantly their physiological relevance, is dependent upon the quality of the bioassays and investigations conducted. Because of inherent limitations of study with human subjects and of in vivo work in general, much investigation has been with in vitro systems for which the suspicion persists that results be unrepresentative of in vivo situations. Many examples of the sort are reviewed here.

Nonetheless, the accumulated in vitro bioassay data and in vivo results support the thesis that oxysterols be toxic agents affecting membrane function and sta-

Table 12. Oxysterol Inhibitions of Cholesterol Biosynthesis from Lanosterol or 5α-Lanost-8-en-3β-ol*

Oxysterol	Inhibition, %	Reference
23,24-Bisnorchol-5-en-3β-ol	6	539
Chol-5-en-3β-ol	3	539
23-Methylchol-5-en-3β-ol	5	539
24-Methyl-26,27-bisnorcholest-5-en-3β-ol	7	539
27-Norcholest-5-en-3β-ol	6	539
26-Methyl-27-norcholest-5-en-3β-ol	2	539
Cholesterol	22	540
(22R)-Cholest-5-ene-3β,22-diol	32	541
(22S)-Cholest-5-ene-3β,22-diol	26	541
Cholest-5-en-3β,25-diol (*7*)	46	540
3β-Hydroxycholest-5-en-7-one (*4*)	25	541
3β-Hydroxy-5α-cholest-8(14)-en-15-one	42–64	158, 541, 542
3β-Hydroxy-14α-methyl-5α-cholest-7-en-15-one	48	542
3β-Hydroxycholest-5-en-24-one	41	541
14α-Methyl-5α-cholest-7-ene-3β,15α-diol	96	542
14α-Ethyl-3β-hydroxy-5α-cholest-7-en-15-one	72	542
14α-Ethyl-5α-cholest-5-ene-3β,15α-diol	93	542
14α-Ethyl-5α-cholest-7-ene-3β,15β-diol	68	542
24,25-Epoxy-(24R)-cholest-5-en-3β-ol	31	541
24,25-Epoxy-(24S)-cholest-5-en-3β-ol (*13*)	54	541
5α-27-Norlanost-8-en-3β-ol	81	540
5α-(24E)-27-Norlanosta-8,24-dien-3β-ol	78	540
5α-26,27-Bisnorlanost-8-en-3β-ol	67	540
5α-26,27-Bisnorlanosta-8,24-dien-3β-ol	53	540
5α-25,26,27-Trisnorlanost-8-en-3β-ol	60	540
5α-24,25,24,27-Tetrakisnorlanost-8-en-3β-ol	51	540
5α-23-Norlanosta-8,24-dien-3β-ol	52	540
15ξ-Fluoro-5α-lanost-7-en-3β-ol	61	542
5α-Lanost-7-en-3β-ol	18	542
5α-Lanost-8-en-3β-ol	17	537
5α-27-Norlanost-8-en-3β-ol	77	537
5α-Lanost-8-ene-3β,7α-diol	47	543
5α-Lanosta-8,24-dien-3α-ol	34	543
5α-Lanosta-8,24-dien-3β-ol	23	543
5α-Lanost-7-ene-3β,15α-diol	96	542
5α-Lanost-7-ene-3β,15β-diol	51	542
5α-(24R)-Lanost-8-ene-3β,24-diol	46	543
5α-(24S)-Lanost-8-ene-3β,24-diol	43	543
5α-Lanost-8-ene-3β,25-diol	64–66	540, 543
5α-(22R)-Lanosta-8,24-diene-3β,22-diol	17	543
5α-(22S)-Lanosta-8,24-diene-3β,22-diol	21	543
5α-Lanosta-8,24-diene-3β,26-diol	75	543
3β-Hydroxy-5α-lanosta-8,24-dien-26-al	0	543
3β-Hydroxy-5α-lanost-8-en-7-one	98	543
3β-Hydroxy-5α-lanost-7-en-15-one	87	542
3β-Hydroxy-5α-lanost-8-en-24-one	43	543
5α-Lanosta-8,24-dien-3-one	16	543
24,25-Epoxy-5α-(24R)-lanost-8-en-3β-ol	55	543
24,25-Epoxy-5α-(24S)-lanost-8-en-3β-ol	61	543
26-Methyl-5α-27-norlanost-8-en-3β-ol	19	537
26-Ethyl-5α-27-norlanost-8-en-3β-ol	9	537
26,26-Dimethyl-5α-27-norlanost-8-en-3β-ol	28	537
26-Propyl-5α-27-norlanost-8-en-3β-ol	8	537
26-2'-Propyl-5α-27-norlanost-8-en-3β-ol	8	537
26-Butyl-5α-27-norlanost-8-en-3β-ol	12	537
26-Pentyl-5α-27-norlanost-8-en-3β-ol	0	537
26-Hexyl-5α-27-norlanost-8-en-3β-ol	0	537

*Tested at 40 μM concentrations in rat liver microsomal S-10 enzyme fraction incubations of lanosterol or 5α-lanost-8-en-3β-ol as substrate.

bility and altering the activity of specific intracellular enzymes, with attendant influences of cell viability, growth, and proliferation. Whether endogenous or dietary oxysterols exert such toxicities in human subjects, causing or exacerbating human health disorders, remains unknown.

However, the toxic properties of select oxysterols are being used as promising guide to creation of potential antitumor agents, and the presence of oxysterols in blood may be regarded as a heretofore unrecognized protection against microbial infection or malignant tumor cell invasion and against the erythrocyte instability of hereditary spherocytosis. On the other hand a growing corpus of experimental work supports the viewpoint that endogenously derived oxysterols be intracellular regulatory agents of de novo sterol biosynthesis. If so, a whole new aspect of oxysterol biochemistry may be revealed, one that might be manipulated to advantage in control of cholesterol metabolism in vivo.

Thus, there are two different hypotheses implicated in these divers results. The more established hypothesis states that dietary and/or endogenously derived oxysterols be cytotoxic and pose threat to human health via chronic exposure to low levels of oxysterols that exert their toxic effects by identified mechanisms (suppression of de novo sterol biosynthesis and by membrane effects) or by other means yet to be recognized. The newer hypothesis states that endogenous oxysterols derived from (3S,22S)-squalene 2,3;22,23-bisepoxide, lanosterol, desmosterol, or cholesterol serve as regulators of de novo sterol biosynthesis via their inhibition of the rate-limiting enzyme HMGCoA reductase, regulation of sterol biosynthesis then presumably being implicated in the established toxic manifestations.

A current distinction offered between these hypotheses is that of the apparent concentration necessary for effect. The suppression of de novo sterol biosynthesis in cultured mammalian cells by endogenous oxysterols occurs at levels considerably below those

exerting toxic effects in vitro. This distinction evinces faith that results with cultured cells have quantitative physiological significance but ignores the complexity of unsuspected in vivo effects in living systems. The question whether low levels of oxysterols present in vivo at levels lower than those demonstrated toxic in vitro but higher than those suggested as regulatory in vitro exert regulatory or chronic toxic effects in vivo needs address, lest physiological relevance be lost to disputation.

The significance, if any, of micromolar levels of several oxysterols in human plasma demands attention. These high levels of oxysterols may enter cells by uptake and internalization of lipoproteins containing oxysterols and by incorporation of plasma oxysterols into the cell plasma membrane from where oxysterol delivery within the cell may occur for sequestration by cytosolic oxysterol binding protein for subsequent regulation, metabolism, excretion, accumulation, or other process. As oxysterol in vivo metabolism, blood transport, fecal excretion, and tissue accumulation are established matters, there remain the questions whether oxysterols be regulatory in vivo and whether toxic oxysterols pose chronic burden to human health.

No adequate work has been reported dealing with chronic exposure to low levels of oxysterols in vivo. No conclusive study of blood oxysterols levels designed to test such levels as an independent risk factor in atherosclerosis or other health disorder has been posed. Accordingly, these matters must await future studies for elucidation if not resolution.

NOTE ADDED IN PROOF

A book dealing with biological activities of oxysterols has been published:
Beck, J. P.; Crastes de Paulet, A., eds. *Activité biologiques des oxystérols*. Paris: INSERM (Institut de la Santé et de la Recherche Médicale); 1987:319 pp.

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A Comparison of Hypocholesterolemic Activity of β-Sitosterol and β-Sitostanol in Rats[1]

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ABSTRACT The hypocholesterolemic activity of β-sitosterol and its hydrogenated product, β-sitostanol (dihydrositosterol or stigmastanol) has been compared in young male rats. When cholesterol was included in the diet, sitostanol consistently exhibited significantly greater hypocholesterolemic activity than sitosterol. There were no apparent differences in the effects of the sterol and the stanol on the concentration of liver cholesterol and triglyceride. Increases in plasma triglyceride due to feeding sitosterol were not observed with sitostanol. Incorporation of dietary sitostanol into plasma, liver and other tissues was always negligible, and thus this stanol was almost completely recovered in feces, while there was considerable deposition of sitosterol (mean fecal recovery being 85% to 92%). The increase in fecal output of dietary cholesterol was significantly greater with the stanol than with the sterol. There was no demonstrable negative effect on growth and weight of major visceral tissues in rats fed the sterol as well as the stanol. These observations together with those reported previously indicate that hydrogenation of phytosterols is a novel approach to enhance their hypocholesterolemic activities without influencing the relative safety of the initial sterols. J. Nutr. *107*: 2011–2019, 1977.

INDEXING KEY WORDS sitosterol · sitostanol · plasma cholesterol

The hypocholesterolemic action of phytosterols in experimental animals and man has been known for many years. β-Sitosterol is a cholesterol-lowering compound of modest to moderate efficacy, and seems remarkably free of subjective side-effects for the patient with hypercholesterolemia (1). Use of a phytosterol mixture containing considerable amount of campesterol is not recommended because of an appreciable incorporation of this sterol into plasma, although there is no evidence that campesterol is more atherogenic than cholesterol (2). Thus, it appears likely that the preference of phytosterols for use in the treatment of hypercholesterolemia depends on their chemical structures. Since there is evidence that plant sterols originating from the diet may initiate development of xanthomatosis and perhaps atheroma (3), low levels of absorption are desirable. On the other hand, few data are available indicating postabsorptive effects of phytosterols (4). Hydrogenation of phytosterols to the corresponding phytostanols results in a significant reduction in absorption (5) similar to the observation with cholesterol and cholestanol (6).

Only limited information is available as to the occurrence of stanols in plant sterol fractions (7). Preliminary experiments showed that commercial unhydrogenated vegetable oils contained demonstrable amounts, up to 23% of the total sterol

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TABLE 1

Effects of β-sitosterol and β-sitostanol on the

Exp No.[a]	Groups (No. of rats)	Dietary fats	Sterols added to the basal diet: Cholesterol	Sitosterol	Sitostanol	Plasma: Cholesterol	Triglycerides
		%	%	%	%	mg/dl[b]	mg/dl[b]
1	1 (6)	Safflower, 5	0	0	0	120 ±11	214 ±30
	2 (6)	Safflower, 5	0	0.5	0	126 ± 6	257 ±33
	3 (6)	Safflower, 5	0	0	0.5	115 ± 5	179 ±13
2	4 (6)	Safflower, 5	0	0	0	98.3± 5.0	138 ±18
	5 (6)	Safflower, 5	0.5	0	0	141 ± 4	126 ±16
	6 (6)	Safflower, 5	0.5	0.5	0	117 ± 4[c]	274 ±30[c]
	7 (6)	Safflower, 5	0.5	0	0.5	95.7± 5.0[a,c]	138 ±23[a,c]
3	8 (6)	Safflower, 5	0	0	0	107 ± 9	121 ±13
	9 (6)	Safflower, 5	0.5	0	0	166 ±12	133 ±21
	10 (6)	Safflower, 5	0.5[d]	0	0	426 ±35	214 ±18
	11 (6)	Safflower, 5	0.5[d]	0.5	0	190 ±19[c]	152 ±14[c]
	12 (6)	Safflower, 5	0.5[d]	0	0.5	166 ± 6[c]	138 ±15[c]
4	13 (7)	Safflower, 5	0.5[d]	1.0	0	120 ± 7	122 ±10
	14 (7)	Safflower, 5	0.5[d]	0	1.0	96.2± 3.5[a]	97.3± 5.2
5	15 (7)	Corn, 5	0.5	0.5	0	97.6± 4.1	138 ±17
	16 (8)	Corn, 5	0.5	0	0.5	80.7± 4.3[a]	111 ± 5[a]
	17 (7)	Lard, 5	0.5	0.5	0	98.3± 5.6	160 ± 9
	18 (8)	Lard, 5	0.5	0	0.5	86.3± 3.4[a]	112 ± 9[a]
6	19 (6)	Safflower, 20	0.5	0	0	83.9± 3.6	67.3± 5.4
	20 (7)	Safflower, 20	0.5	0.5	0	77.1± 3.6	102 ± 5[c]
	21 (7)	Safflower, 20	0.5	0	0.5	59.0± 1.2[a,c]	78.2± 6.8[a]
	22 (6)	Lard, 20	0.5	0	0	87.3± 3.0	81.1± 5.0
	23 (7)	Lard, 20	0.5	0.5	0	75.3± 1.7[a]	133 ± 8[c]
	24 (7)	Lard, 20	0.5	0	0.5	66.7± 3.0[a,c]	90.8± 6.4[a]

fraction was stanols. The corresponding value for β-sitosterol preparations was 10% to 15%. Since our previous experiments demonstrated that in rats, the hypocholesterolemic action of phytostanol mixtures was demonstrably greater than that of phytosterol mixtures (5), a comparison between β-sitosterol and β-sitostanol was made in the present study.

MATERIALS AND METHODS

Materials. β-Sitosterol[1] was repeatedly recrystallized from ethyl acetate until over 98% purity as the sterols was achieved as measured by gas-liquid chromatography (GLC). The composition of the sterol fraction of this preparation was 93% β-sitosterol and 7% campesterol. Capillary column GLC revealed the occurrence of stanols in this preparation (0.5% campestanol and 12.5% sitostanol). Hydrogenation of β-sitosterol was performed as reported previously (5). The purity of the product was checked by nuclear magnetic resonance spectrometry,[2] by Ag$^+$-thin-layer chromatography (TLC) (8) and by GLC on a glass capillary column (5). Judging from GLC and TLC analyses, the hydrogenated compound still contained approximately 3% of the unhydrogenated sterols, and the apparent composition of the reduction products was 93% β-sitostanol and 7% campestanol.

Animals and diets. Male Wistar rats obtained from a commercial breeder[3] and weighing 85 to 115 g were used throughout the experiment. The composition of the basal diet was (in %) (5): vitamin free casein,[4] 20; mineral mixture, 4; vitamin mixture, 1; choline chloride, 0.15; cellulose powder,[5] 4 and sucrose to 100. The vitamin (water soluble) and mineral mixtures were according to Harper (9).[6] Fats, cholesterol,

[1] E. Merck, Darmstadt.
[2] Japan Electron Optic Laboratory JNM-MH 100 NMR spectrometer, Tokyo.
[3] Kyudo Co., Kumamoto.
[4] ICN Pharmaceuticals Inc., Cleveland, Ohio.
[5] Toyo Kagaku Sangyo Co., cellulose powder Type E, Tokyo.
[6] The Tanabe Amino Acid Research Foundation, Osaka.

concentration of plasma and liver lipids

Plasma			Liver					
Cholesterol	Campesterol	Sitosterol	Weight	Cholesterol	Triglycerides	Cholesterol	Campesterol	Sitosterol
% of total sterols[2]			g/100 g body wt.[3]	mg/g[3]	mg/g[3]	% of total sterols[2]		
98.0±0.3	0.5±0.1	1.5±0.3	4.48±0.11	2.52±0.08	17.6±1.1	98.4±0.1	0.8±0.1	0.8±0.1
98.4±1.0	1.0±0.2	0.3±0.5	4.77±0.22	2.25±0.06	19.6±1.5	89.7±0.6	2.6±0.1	8.2±0.5
97.5±0.2[a]	0.5±0.1[a]	2.0±0.2[a]	4.13±0.12	2.47±0.10	16.8±0.4	97.7±0.2[a]	0.6±0.1[a]	1.6±0.2[a]
98.3±0.2	0.5±0.1	0.9±0.1	4.40±0.26	3.15±0.20	17.7±1.5	98.7±0.1	0.7±0.1	0.7±0.1
99.6±0.1	0.2±0.0	0.2±0.1	4.90±0.12	22.5 ±2.0	41.4±2.2	100	tr[4]	tr
93.5±0.6[c]	0.9±0.1[c]	5.5±0.9[c]	4.62±0.18[d]	4.00±0.24[c]	22.6±2.6[c]	96.5±0.5[c]	0.8±0.1[c]	3.2±0.5[c]
98.6±0.1[c,d]	0.4±0.0[c,d]	1.0±0.1[c,d]	4.26±0.07[c,d]	4.09±0.21[c]	18.2±0.8[c]	98.9±0.2[a]	0.6±0.1[c]	0.5±0.1[c]
99.2±0.1	0.3±0.0	0.5±0.0	4.27±0.06	2.94±0.08	17.2±0.9	99.1±0.0	0.4±0.0	0.5±0.0
100	tr	tr	4.60±0.20	23.1 ±1.0	28.9±2.0	100	tr	tr
100	tr	tr	5.36±0.11	43.6 ±1.7	55.9±2.6	100	tr	tr
97.8±0.2[c]	0.6±0.0[c]	1.6±0.2[c]	4.63±0.14[c]	17.0 ±0.9[c]	28.8±2.9	99.1±0.1[c]	0.4±0.0[c]	0.5±0.1[c]
99.4±0.1[a]	0.2±0.0[a]	0.4±0.1[a]	4.60±0.08[c]	14.0 ±1.0[a,c]	26.8±1.9	99.5±0.0[a]	0.1±0.0[a]	0.1±0.0[a]
96.0±0.6	1.2±0.1	2.8±0.5	4.21±0.09	16.3 ±1.8	38.3±2.7	97.6±0.3	1.2±0.3	0.9±0.2
99.3±0.1[a]	0.4±0.1[a]	0.3±0.1[a]	4.00±0.10	12.7 ±1.0	26.6±1.6[a]	99.4±0.1[a]	0.5±0.1[a]	0.0±0.0[a]
93.4±0.5	1.6±0.1	5.0±0.4	3.95±0.10	5.20±0.28	25.4±1.7	97.0±0.3	1.1±0.1	1.9±0.5
97.1±0.1[a]	1.1±0.0[a]	1.8±0.1[a]	3.52±0.03[a]	4.51±0.30	27.0±1.1	99.0±0.3[a]	0.5±0.2[a]	0.5±0.1[a]
93.1±0.5	1.4±0.1	5.4±0.5	3.77±0.10	4.51±0.30	19.5±0.7	97.1±0.3	0.9±0.1	2.1±0.2
99.0±0.1[a]	0.4±0.0[a]	0.6±0.0[a]	3.50±0.10	5.01±0.25	20.7±1.3	99.6±0.1[a]	0.3±0.0[a]	0.1±0.0[a]
98.7±0.1	0.7±0.0	0.6±0.1	3.94±0.13	50.1 ±2.2	129 ±4	99.5±0.0	0.1±0.0	0.1±0.0
95.3±0.3[c]	1.5±0.1[c]	3.2±0.2[c]	3.51±0.08[d]	20.7 ±1.9[c]	83.1±2.0[c]	99.2±0.1[c]	0.3±0.0[c]	0.6±0.0[c]
98.9±0.1[a]	0.5±0.0[a]	0.6±0.0[a]	3.20±0.07[c]	20.6 ±1.1[c]	70.9±3.2[c]	99.5±0.0[a]	0.1±0.0[a]	0.1±0.0[a]
99.7±0.0	0.2±0.0	0.1±0.0	4.06±0.15	61.2 ±1.6	99.5±4.0	99.9±0.0	0.1±0.0	tr
95.2±0.2[c]	1.3±0.1[c]	3.5±0.1[c]	3.53±0.08[d]	23.0 ±2.5[c]	70.3±4.6[c]	99.0±0.1[c]	0.4±0.1[c]	0.6±0.1[c]
99.1±0.1[a]	0.3±0.1[a]	0.4±0.1[a]	3.32±0.07[a,c]	23.9 ±1.1[c]	67.3±1.9[c]	99.8±0.0[a]	0.1±0.0[a]	0.1±0.0[a]

[1] Feeding periods: Exp. 1 and 2 for 23 days, exp. 3 for 14 days and exp. 4 to 6 for 21 days. Initial body weights: exp. 1, 112 g; exp. 2, 94 g; exp. 3, 85 g; exp. 4, 91 g; exp. 5, 112 g and exp. 6, 111 g. Final body weights: group 1, 150±7 g; group 2, 178±8 g; group 3, 163±7 g; group 4, 225±13 g; group 5, 248±16 g; group 6, 257±16 g; group 7, 237±16 g; group 8, 183±4 g; group 9, 179±4 g; group 10, 186±4 g; group 11, 179±4 g; group 12, 183±4 g; group 13, 222±6 g; group 14, 215±8 g; group 15, 238±6 g; group 16, 241±8 g; group 17, 244±8 g; group 18, 246±4 g; group 19, 239±9 g; group 20, 234±9 g; group 21, 232±6 g; group 22, 255±6 g; group 23, 252±9 g, and group 24, 257±4 g. [2] Mean ±SEM. [3] Trace. [4] Cholic acid (0.125%) was added simultaneously. [a] Significantly different from the corresponding sitosterol fed group at $P < 0.01$. [b] Significantly different from the corresponding sitosterol fed group at $P < 0.05$. [c] Significantly different from the corresponding group fed cholesterol alone or cholesterol plus cholic acid at $P < 0.01$. [d] Significantly different from the corresponding group fed cholesterol alone or cholesterol plus cholic acid at $P < 0.05$.

sitosterol and sitostanol were added at the expense of sucrose. Routinely, safflower oil was used as a dietary fat and to this were added retinyl palmitate, 400 IU; cholecalciferol, 200 IU and *dl*-α-tocopherol, 10 mg/100 g of diet. The content of sterols in dietary fats as determined by GLC and using 5α-cholestane as a calibration standard was as follows (in %): safflower oil, 0.18 to 0.22, corn oil, 0.52 and lard, 0.01 to 0.04.

The dietary regimens are shown in table 1. Sterols were finely dispersed in warm dietary fats prior to mixing them with other ingredients. Rats were given free access to the diets and water, and killed after fasting overnight (18:00 to 09:00 hours). Body weight and food consumption were recorded every other day. Feces were collected for 2 days beginning 5 days before killing the rats. During this time, the diets contained 0.1% of Cr_2O_3. Fecal recovery of sterols was calculated on the basis of analyses of sterols and Cr in feces and diets.

Lipid analyses. Rats were fasted overnight and killed by decapitation. Analyses of lipid components of plasma and liver were performed as described previously (5). The presence of the sterols or stanols, even at the same concentration as that of cholesterol, did not influence the accuracy

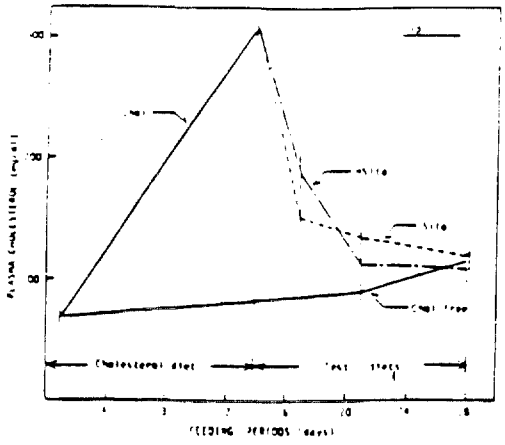

Fig. 1 Effect of dietary β-sitosterol and β-sitostanol on the concentration of plasma cholesterol in rats previously fed the high cholesterol diet (exp. 7). Rats weighing an average of 115 g were fed the hypercholesterolemic diet (0.5% cholesterol plus 0.125% cholic acid) for 14 days, and then one of the diets containing either 0.25% of sitosterol or sitostanol for 3, 7, and 14 days. One group of rats was fed the sterol free diet throughout. Each point represents mean ± SEM of 5 rats per group. Chol: cholesterol, Sito: sitosterol and HSito: sitostanol.

of cholesterol determination by the Liebermann-Burchard reaction of the digitonide. GLC [9] of the trimethyl silyl ether of the unsaponifiable fraction was performed on a 3% OV-17 glass column [10] (5, 10). For separation of sterols and stanols, GLC and Ag^+-TLC were employed; a gas chromatograph equipped with a Polyimide 100 glass-WCOT column, 0.25 mm × 50 m [11] was used. The apparatus was operated at 260° with carrier gas (N_2) at 1.0 ml/minute; under this condition separation of sterols and stanols was complete. Ag^+-TLC was performed using Silica gel G^1 plate containing $AgNO_3$ (25%, w/w) and developing twice in chloroform-methanol (99.8:0.2, v/v) (8).

A portion of lyophilized feces was extracted with ethanol in Soxhlet apparatus for 30 hours. The extract was saponified and the trimethyl silyl ether derivative formed and analyzed by GLC (5, 10). A known amount of 5α-cholestane [12] in ethanol was added to the feces prior to extracting sterols with ethanol as an internal calibration standard. Cr in diets and feces was determined colorimetrically after reducing them to ashes.

Statistical analysis. Data were analyzed by the Student's *t* test (11).

RESULTS

Growth and tissue weights. There were no differences in the food intake or weight gains between rats fed the sterol or the stanol in any experiment. Although the weights of major viscera, (kidney, heart, lung, spleen, adrenal and testes) were also similar for these two groups, liver weight appeared somewhat lower in the stanol fed rats when cholesterol was simultaneously included in the diet, some of the differences were statistically significant (table 1).

Plasma and liver lipid concentration. Table 1 summarizes the concentration of plasma and liver cholesterol and triglyceride. The data reported as cholesterol were derived from the Liebermann-Burchard reaction of the digitonide. Since phytosterols develop color, but to a considerably weaker degree than cholesterol does, the correction for this seemed necessary, in particular in those samples containing detectable amounts of phytosterols. The correction did not modify the statistical significance listed in this table. Essentially no color was developed with the stanol digitonides by this reaction.

When cholesterol was simultaneously added to the diet, the hypocholesterolemic activity of the stanol was at all times significantly greater than that of the sterol, except for experiment 3 where cholic acid was also added to the diet. (In this case, plasma concentration of cholesterol tended to be lower with the stanol, but the difference was not significant.) Also, the rise in the concentration of plasma triglyceride after feeding the sterol was not observed with the stanol feeding.

Inclusion of the stanol in the diet at the same level as that of cholesterol lowered the plasma cholesterol to the levels that were apparently similar to those of rats fed no cholesterol (exp. 2). A similar trend was also observed in rats fed twice as much sterol as cholesterol in the presence of cholic acid (exp. 4).

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[9] Japan Electron Optic Laboratory JGC-50F gas chromatograph, Tokyo.
[10] Applied Science Laboratories, Inc., Inglewood, Calif.
[11] Shimadzu GC-4CMPF gas chromatograph, Kyoto.
[12] Sigma Chemical Co., St. Louis, Missouri.

The concentration of plasma triglycerides in rats fed sitostanol alone tended to be lower than that of rats fed sitosterol alone, but the difference was not statistically significant (exp. 1).

The effects of different sources of dietary fats were examined. In experiment 5, where corn oil or lard was added to the diet at the 5% level, the responses of plasma cholesterol to the sterol and the stanol resembled those observed in the preceding experiments in which safflower oil was used as a dietary fat source at the same level. Similar results were obtained in rats fed high fat diets (exp. 6) and the stanol feeding resulted in a significant reduction in plasma cholesterol levels compared to those of rats fed cholesterol alone ($P < 0.001$), while with the sterol the difference was significant only in rats fed lard ($P < 0.05$).

In all experiments, the concentration of unesterified cholesterol was simultaneously determined. When elevated plasma levels of cholesterol in cholesterol-fed rats were lowered nearly to or below the control values by supplementing either the sterol or the stanol, the ratio of free to esterified cholesterol also became normal.

In one experiment (exp. 7), rats were fed a high cholesterol diet (0.5% cholesterol and 0.125% cholic acid) for 2 weeks and followed by a cholesterol-cholic acid free diet containing sitosterol or sitostanol at the 0.25% level for an additional 2 weeks. When the cholesterol diet was replaced by the sitosterol or sitostanol diet, the concentration of plasma cholesterol sharply decreased to level corresponding to that for control rats fed the cholesterol free diet throughout (fig. 1). Although the initial fall in the plasma cholesterol during the 3 days after switching the diet appeared markedly greater with sitosterol, the final level after 2 weeks tended to be lower with sitostanol. In addition, the concentration of plasma triglyceride continued to fall as a result of feeding sitostanol, while that of rats fed sitosterol remained high (fig. 2). Thus, 2 weeks after changing diets, the triglyceride level in sitosterol fed rats was significantly higher than that of rats fed sitostanol.

As table 1 shows, the extent of reduction of hepatic cholesterol and triglyceride by the sterol and the stanol appeared similar, although in some cases there were significant reductions of liver lipid levels due to feeding the stanol compared to the sterol.

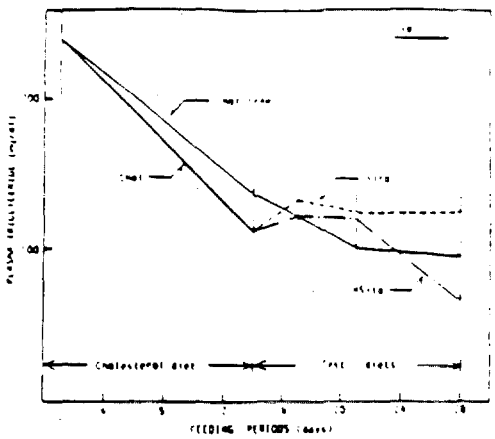

Fig. 2 Effect of dietary β-sitosterol and β-sitostanol on the concentration of plasma triglyceride in rats previously fed the high cholesterol diet (exp. 7). Each point represents mean ± SEM of 5 rats per group. Chol: cholesterol, Sito: sitosterol and HSito: sitostanol. $P < 0.05$ for sitosterol versus sitostanol at the termination of the experiment.

Sterol composition of plasma, liver and other tissues. Table 1 summarizes the percent composition of sterols in the unsaponifiable fractions from plasma and liver. It is obvious that rats fed sitostanol, in comparison to those fed sitosterol, had significantly lower percentage of the sterols other than cholesterol. Changes in the amounts and compositions of dietary fats did not influence this observation. It was also apparent that both plasma and liver contained approximately similar patterns of percentage of plant sterols.

Analyses of the sterols by Ag^+-TLC and capillary column GLC revealed that after feeding the stanol less than 3% of the plant sterols detected was the hydrogenated compounds. No hydrogenated cholesterol was detected. Thus, almost all of the plant sterols detected in rats fed the stanol were the sterols, and most likely originated from the sterols contaminating the stanol preparation and from those occurring in the dietary fats. The latter assumption could be supported by the observation that rats fed corn oil, which contained twice as much plant sterols as safflower oil, contained a

TABLE 2

Effect of β-sitosterol and β-sitostanol on the composition of epididymal adipose tissue and adrenal sterol

Exp No	Groups (No. of rats)	Sterols added to the basal diet			Adipose tissue			Adrenals		
		Cholesterol	Sitosterol	Sitostanol	Cholesterol	Campesterol	Sitosterol	Cholesterol	Campesterol	Sitosterol
		%	%	%	% of total sterols[1]			% of total sterols[1]		
3	8 (6)	0	0	0	96.7	1.2	2.1	ND[2]	ND	ND
	9 (6)	0.5	0	0	98.8	0.6	0.6	ND	ND	ND
	10 (6)	0.5[3]	0	0	99.2	0.5	0.3	ND	ND	ND
	11 (6)	0.5[3]	0.5	0	94.5	1.4	4.1	ND	ND	ND
	12 (6)	0.5[3]	0	0.5	98.6	0.4	1.0	ND	ND	ND
4	13 (7)	0.5[3]	1.0	0	95.5	1.2	3.3	96.0 ±0.4[4]	1.3 ±0.1	2.8 ±0.3
	14 (7)	0.5[3]	0	1.0	99.2	0.3	0.5	98.8 ±0.1[5]	0.5 ±0.0[5]	0.5 ±0.1[5]
5	15 (7)	0.5	0.5	0	95.2	1.1	3.7	95.6 ±0.5	0.9 ±0.1	3.6 ±0.5
	16 (8)	0.5	0	0.5	98.5	0.5	1.0	99.3 ±0.1[5]	0.3 ±0.0[5]	0.4 ±0.1[5]
	17 (7)	0.5	0.5	0	95.7	0.7	3.6	96.4 ±0.4	1.1 ±0.4	2.5 ±0.4
	18 (8)	0.5	0	0.5	99.1	0.4	0.5	98.3 ±0.1[5]	0.7 ±0.0[5]	1.0 ±0.1[5]
6	19 (6)	0.5	0	0	98.7	0.4	0.9	98.6 ±0.1	0.8 ±0.1	0.7 ±0.1
	20 (7)	0.5	0.5	0	95.3	1.1	3.6	95.8 ±0.3[6]	1.2 ±0.1[6]	3.0 ±0.2[6]
	21 (7)	0.5	0	0.5	98.2	0.5	1.3	98.8 ±0.1[5]	0.6 ±0.0[5]	0.6 ±0.0[5]
	22 (6)	0.5	0	0	98.9	0.3	0.8	99.7 ±0.0	0.2 ±0.0	0.1 ±0.0
	23 (7)	0.5	0.5	0	94.9	1.1	4.0	95.1 ±0.4[6]	1.4 ±0.1[6]	3.5 ±0.3[6]
	24 (7)	0.5	0	0.5	98.5	0.4	1.1	99.1 ±0.0[5]	0.4 ±0.1[5]	0.4 ±0.1[5]

[1] Values are from pooled samples. [2] Mean ±SEM. [3] Not determined. [4] Cholic acid (0.125%) was added simultaneously. [5] Significantly different from the corresponding sitosterol fed group at $P < 0.01$. [6] Significantly different from the corresponding group fed cholesterol alone at $P < 0.01$.

higher percentage of the plant sterols than those fed safflower oil. This observation, together with that from the sterol balance studies which will be described later, clearly supports the view that only negligible amounts of the stanols are incorporated into the body. Similar results were obtained with adipose tissue and adrenal glands (table 2).

Table 3 shows the concentration and composition of aorta sterols. Again there was demonstrable deposition of plant sterols, but not of the stanols, into the aorta. Though these data were from pooled samples, the concentration of cholesterol appeared somewhat lower in rats of the two groups fed the sterol or the stanol plus cholesterol than that of rats fed cholesterol alone.

Fecal recovery of cholesterol, sitosterol and sitostanol. These data were derived from the ratios of Cr and sterols in the diets

TABLE 3

Effect of β-sitosterol and β-sitostanol on the concentration and composition of aorta sterol

Exp No.	Groups (No. of rats)	Sterols added to the basal diet			Total sterols	Sterol composition		
		Cholesterol	Sitosterol	Sitostanol		Cholesterol	Campesterol	Sitosterol
		%	%	%	mg/100 mg[1]	% of total sterols[2]		
6	19 (6)	0.5	0	0	0.18	99.5	0.2	0.3
	20 (7)	0.5	0.5	0	0.17	98.6	0.5	0.9
	21 (7)	0.5	0	0.5	0.17	99.5	0.3	0.2
	22 (6)	0.5	0	0	0.18	99.8	0.1	0.1
	23 (7)	0.5	0.5	0	0.17	98.6	0.4	1.0
	24 (7)	0.5	0	0.5	0.16	99.5	0.3	0.2

[1] Values are from pooled samples. [2] Total sterols were determined by GLC using 5α-cholestane as an internal calibration standard.

to those in the feces, and were corrected for endogenous excretion in the corresponding cholesterol free control group.

As Table 4 shows, fecal recovery of added cholesterol was consistently and significantly greater in rats fed sitostanol than those fed sitosterol. In rats fed no cholesterol, average excretion of cholesterol (includes its coprostanol derivative) was 0.42 mg/100 mg dried feces and that of plant sterol (includes its coprostanol derivative) was 0.20 mg/100 mg. When no cholesterol was included in the diet (exp. 1), sitostanol also tended to stimulate excretion of endogenous cholesterol more than did sitosterol (1.76 times versus 1.46 times in comparison with control rats), but the difference was not significant. This may be due to the limited quantity of endogenous neutral sterols excreted by rats fed the diet [illegible] of cholesterol.

In addition, irrespective of the presence or absence of cholesterol, sitostanol added to the diets was almost completely recovered in the feces. The average fecal recovery of dietary sitosterol ranged between 85% to 92%. Addition of cholic acid to the cholesterol containing diets did not affect these results, nor did the quantity and the quality of dietary fats. The somewhat lower recovery, in comparison to the reported data (2), of sitosterol in these experiments may in part be due to the way by which this sterol was mixed into the diets. From the data shown in table 1, it was clear that significant amounts of sitosterol were absorbed.

DISCUSSION

The ideal hypocholesterolemic agent should be effective, and free of subjective side-effects and of toxicity. Plant sterols come close to that ideal (12). Although one major advantage of the use of phytosterols for treatment of hypercholesterolemia is that they are relatively safe (1), it has been generally believed that relatively large doses of them are required to obtain a sig-

TABLE 4

Excretion of Cholesterol, β-sitosterol and β-sitostanol

Exp No.	Groups (No. of rats)	Sterols added to the basal diet			Fecal excretion		
		Cholesterol	Sitosterol	Sitostanol	Cholesterol	Sitosterol	Sitostanol
		%	%	%	%[1]	%[1]	%[1]
1	2 (6)	0	0.5	0	—	85.3±2.1	—
	3 (6)	0	0	0.5	—	—	99.9±1.2[c]
2	5 (6)	0.5	0	0	55.0±1.5	—	—
	6 (6)	0.5	0.5	0	75.3±1.5	88.5±3.1	—
	7 (6)	0.5	0	0.5	82.9±1.5[a]	—	101 ±2[c]
3	10 (6)	0.5[2]	0	0	25.5±0.7	—	—
	11 (6)	0.5[2]	0.5	0	61.6±2.0	86.5±0.9	—
	12 (6)	0.5[2]	0	0.5	76.2±1.0[b]	—	100 ±2[c]
4	13 (7)	0.5[2]	1.0	0	73.0±2.1	86.6±1.8	—
	14 (7)	0.5[2]	0	1.0	86.2±1.9[b]	—	100 ±1[c]
5	15 (7)	0.5	0.5	0	75.3±0.6	88.9±2.3	—
	16 (8)	0.5	0	0.5	87.1±3.2	—	97.2±1.4[d]
	17 (7)	0.5	0.5	0	64.3±1.1	91.9±2.1	—
	18 (8)	0.5	0	0.5	85.1±1.9[b]	—	100 ±3[c]
6	19 (6)	0.5	0	0	34.6±1.4	—	—
	20 (7)	0.5	0.5	0	60.4±0.9	87.1±3.2	—
	21 (7)	0.5	0	0.5	77.3±1.9[b]	—	100 ±2[c]
	22 (6)	0.5	0	0	30.1±1.1	—	—
	23 (7)	0.5	0.5	0	66.5±1.8	86.5±1.3	—
	24 (7)	0.5	0	0.5	83.0±1.4[b]	—	101 ±1[c]

[1] Mean±SEM. [2] Cholic acid (0.125%) was added simultaneously. [a] Significantly different from the corresponding sitosterol fed group at $P < 0.05$. [b] Significantly different from the corresponding sitosterol fed group at $P < 0.01$. [c] Difference between sitosterol and sitostanol was significant at $P < 0.01$. [d] Difference between sitosterol and sitostanol was significant at $P < 0.05$. Difference in excretion of cholesterol between rats fed cholesterol alone and cholesterol plus sitosterol or sitostanol was significant at $P < 0.001$.

nificant cholesterol lowering effect. This has been considered to be a major disadvantage of the use of these sterols as therapy for hypercholesterolemia. A recent study by Grundy and Mok (13) showed that intakes of β-sitosterol as low as 3 g/day inhibited absorption of cholesterol to a maximum degree, and much larger doses did not cause a further significant decrease in absorption of cholesterol. From this finding, these authors consider that phytosterols, in a sense, can be a form of diet therapy rather than drug treatment (13). On the other hand, because of differences in absorbability among individual phytosterols normally encountered in edible oils, the preparations that contain detectable amounts of campesterol can not be recommended for therapeutic use (2). Intestinal absorption of natural plant sterols appears to be affected by the length of the side chain of the molecules, namely, the longer the side chain, the less the absorption (1). Thus, less sitosterol is absorbed than campesterol. In addition, absorption of dihydrosterols appears to be much lower than that of the corresponding sterols, as can be demonstrated with cholesterol and cholestanol (6) as well as phytosterols and phytostanols (5).

On the basis of these considerations, we have examined the hypocholesterolemic activity of nearly pure β-sitosterol and β-sitostanol. Commercial vegetable oils contained varying amounts of the stanols in their so called sterol fractions (unpublished observations). Commercial β-sitosterol preparations from different suppliers also were found to contain 10% to 15% sitostanol. Thus, it seemed likely that β-sitostanol is as safe as β-sitosterol. This assumption is supported by the observation that rats fed phytostanol mixtures (1) or sitostanol grew normally and had normal visceral weights (liver, kidney, heart, lung, spleen, testes and adrenals) and appearance. Furthermore, feeding the stanol never resulted in any apparent abnormality in fecal excretion such as constipation or diarrhea. There were also no differences in the absorbability of dietary protein, sugar and fats (measured in expt. 3).

Sterol balance data shown in table 4 clearly demonstrated that the hydrogenated sterol stimulated cholesterol excretion more than did the unhydrogenated sterol and that the stanol itself was excreted almost completely into feces. The fecal recovery of labeled sitostanol during the first 7 days after oral administration was also almost complete and that of sitosterol was below 90% (data to be published). Transformation of cholesterol to coprostanol was slightly inhibited by the simultaneous ingestion of sitostanol in comparison to that found when sitosterol was ingested (the ratio of coprostanol to cholesterol, 14.5 to 19.4% versus 8.8 to 18.8%). Also, conversion of sitostanol to the corresponding coprositostanol was significantly lower than that of sitosterol (the ratio of coprositostanol to sitosterol or sitostanol, 11.2 to 13.1% versus 1.9 to 3.7%) and this transformation was markedly reduced by the simultaneous ingestion of cholic acid (2.1 to 3.6% versus 1.7 to 2.0%). Since a recent study by Govind Rao et al. (4) suggested probable side-effects of absorbed phytosterols, the observations that sitostanol is essentially not absorbed nor deposited in the body pool and that it possesses a greater cholesterol lowering activity suggest that sitostanol is a more ideal hypocholesterolemic compound than sitosterol. The apparent lack of significant differences in the hypocholesterolemic activity of the sterol and the stanol in rats fed no cholesterol (expt. 1 and also exp. 7) may primarily be due to the relative shortness of the feeding periods. In fact, the concentration of plasma cholesterol in rats fed the stanol in these experiments tended to be slightly lower than that of those eating the sterol. For further validation of the effectiveness of sitostanol for treatment of hypercholesterolemia, we are currently studying their effect on rabbits and the fate of labeled stanol in vivo in rats.

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THE EFFECT OF SITOSTANOL ESTER MARGARINE ON SERUM LIPIDS IN HEALTHY CHILDREN ALREADY ON A DIET LOW IN SATURATED FAT AND CHOLESTEROL. THE STRIP PROJECT

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Sitostanol ester margarine lowers serum cholesterol in a mildly hypercholesterolemic adult population and children with familial hypercholesterolemia. To study the cholesterol lowering effect and tolerability of sitostanol ester margarine in healthy children we launched a study with double blind cross over design. The study subjects were advised to replace 20g per day of their dietary fat with study margarine (sitostanol margarine or placebo) for three months. After six weeks wash-out period the diets were switched over for three months. The study subjects were six years old children taking part in the STRIP project which is a randomized, prospective trial aimed to reduce the predisposition of children to known atherosclerosis risk factors. All the children belonged in the intervention group and, thus, were already on a diet low in saturated fat and cholesterol. Seventy-two children completed the study. Fasting serum total and HDL cholesterol, triglycerides, creatinine, lactic dehydrogenase, and glutamyl transferase were measured by an enzymatic method. LDL cholesterol was calculated according to Friedewald's formula. Red and white blood cells counts, hemoglobin concentration, and red blood cell indices were determined using automatic analyzer. Sitostanol margarine was well tolerated. The mean consumption of study margarine was 18.2 g per day during both the sitostanol and the placebo periods. Consequently, the sitostanol dose was 1.46 g per day. The mean serum total cholesterol concentration prior to placebo and sitostanol periods was 4.35 and 4.26 mmol/L and at the end of periods 4.23 and 4.00 mmol/L, respectively. The corresponding values for LDL cholesterol were 2.74 and 2.65 mmol/L prior to the periods and 2.60 and 2.40 mmol/L at the end. P value for the treatment effect was 0.0003 in serum total cholesterol and 0.0001 in LDL cholesterol. Neither serum HDL cholesterol and triglyceride concentration nor safety markers were affected by sitostanol. We conclude that sitostanol ester margarine has a small but significant decreasing effect on serum total and LDL cholesterol in healthy children who are already on a diet low in saturated fat and cholesterol.

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DATE	29.1.1999	E-mail: paula.virtanen@raisiogroup.com
PAGES	1+ 4	

Dear Dr Kaplan,

You have requested better copy of the Strip study -poster (Tammi's study with children) Poster was presented in EAS meeting and you have copy of the abstract and poster. Enclosed info presented in poster in separate sheets. It was not possible to get better copy of the poster itself, soTammi kindly gave us the "poster" in separate sheets.

Tammi have corrected some figures to table 2. Now in table 2 there is some corrections in campesterol and Δ8-cholestenol values and -to cholesterol ratios. Ratios are now presented as 10^2 μmol/mmol of cholesterol. In original poster ratios were presented as μg/mmol of cholesterol.

These poster sheets delivered also to Arent Fox/ Edwards. I will send these pages also by mail to get better copies to you.

Have a nice weekend !

With best regards, Paula Virtanen

Paula Virtanen
Research Assistant

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THE EFFECT OF SITOSTANOL ESTER MARGARINE ON SERUM LIPIDS IN HEALTHY CHILDREN ALREADY ON A DIET LOW IN SATURATED FAT AND CHOLESTEROL THE STRIP PROJECT

A. Tammi, T. Rönnemaa, H. Gylling, K. Pulkki, O. Simell and the STRIP Study Group
Cardiorespiratory Research Unit and Departments of Pediatrics and Medicine of Turku University, Department of Medicine of Helsinki University, and Department of Laboratory of Turku University Central Hospital, Finland

INTRODUCTION

Atherosclerosis begins early in life. High total and LDL cholesterol concentrations are major risk factors for atherosclerosis and, already in childhood, LDL cholesterol values correlates with the extent of early atherosclerotic lesions. The mean cholesterol concentration of 4.5 mmol/L in Finnish children is higher than in most western countries and higher than recommended. Dietary interventions in healthy or hypercholesterolemic children have achieved inconsistent reduction in serum cholesterol values. Sitostanol ester margarine lowers serum total cholesterol by 10 percent and LDL cholesterol concentration by 15 percent in a mildly hypercholesterolemic adult population and children with familial hypercholesterolemia (FH). Since the benefit of serum cholesterol reduction in atherosclerosis prevention may be especially great when starting at an early age, we wanted to study the cholesterol lowering effect and tolerability of sitostanol ester margarine in healthy six years old children already on a diet low in saturated fat and cholesterol.

SUBJECTS AND METHODS

This study is a part of the STRIP project, which is a randomized, prospective trial aimed at decreasing exposure of young children to known atherosclerosis risk factors. A total of 1062 infants at seven months of age were randomized to intervention (n=540) and control (n=522) groups. Intervention children achieved dietary counseling with main emphasis on a diet low in saturated fat and cholesterol. Eighty-one healthy children aged six years from intervention group were recruited to sitostanol margarine study. The children were advised to replace 20 g per day of dietary fat with study margarine with or without sitostanol ester for three months. After six weeks' wash-out period the diets were switched over for three months. Study was performed double-blinded.
Fasting serum total and HDL cholesterol, triglycerides, creatinine, lactate dehydrogenase and glutamyltransferase were measured by enzymatic methods. LDL cholesterol was calculated according to Friedewald's formula. Red and white blood cell counts, red blood cell indices and hemoglobin concentration were determined using an automatic analyzer. Serum α-tocopherol and β-carotene concentrations were measured with high performance liquid chromatography. Serum noncholesterol sterols were determined with gas chromatography.

RESULTS

Seventy-two children completed the study. Sitostanol ester margarine was well tolerated and no adverse clinical effects were observed. The mean consumption of margarine was 18.2 g during both study periods. Consequently, the sitostanol dose was 1.46 g per day. Sitostanol ester margarine reduced serum total and LDL cholesterol by 5.2 and 7.3 per cent from baseline values, respectively (fig). As HDL cholesterol was not affected, HDL/total cholesterol ratio increased 5.6 percent. Serum triglyceride concentration was unchanged as well as safety markers. Serum α-tocopherol and β-carotene levels decreased slightly. These antioxidants are carried in LDL particles in plasma. Because LDL cholesterol concentration decreased, we calculated α-tocopherol and β-carotene per LDL cholesterol. LDL cholesterol adjusted α-tocopherol was unchanged and LDL cholesterol adjusted β-carotene decreased by 17.6 percent (table 1). Serum campesterol concentration as well as campesterol to cholesterol ratio were reduced by sitostanol treatment (table 2). The reduction in serum total and LDL cholesterol

concentrations showed positive correlations with the reduction in serum campesterol concentration ($r=0.42$, $p=0.0001$ and $r=0.39$, $p= 0.0005$,respectively). Concentration of cholesterol precursor sterol, $\Delta 8$-cholestenol, showed an increase (table 2).

CONCLUSIONS

Sitostanol ester margarine has a significant decreasing effect on serum total and LDL cholesterol concentrations in healthy children who are already on a diet low in saturated fat and cholesterol. The decrease in serum total and LDL cholesterol concentrations observed in this study was smaller than the respective decrease in studies performed in adults or in children with FH. However, our study subjects showed lower baseline cholesterol values than the others. This might contribute to our result. No adverse effects were observed in our short term study. The significance of a reduction in β-carotene/LDL ratio is unknown. A marked reduction in serum campesterol concentration suggests that a decrease in serum cholesterol values is due to inhibition of cholesterol absorption caused by sitostanol ingestion. An increase in serum $\Delta 8$-cholestenol reflects compensatory activation in serum cholesterol synthesis.

Serum lipids before and after three months' sitostanol and control study periods

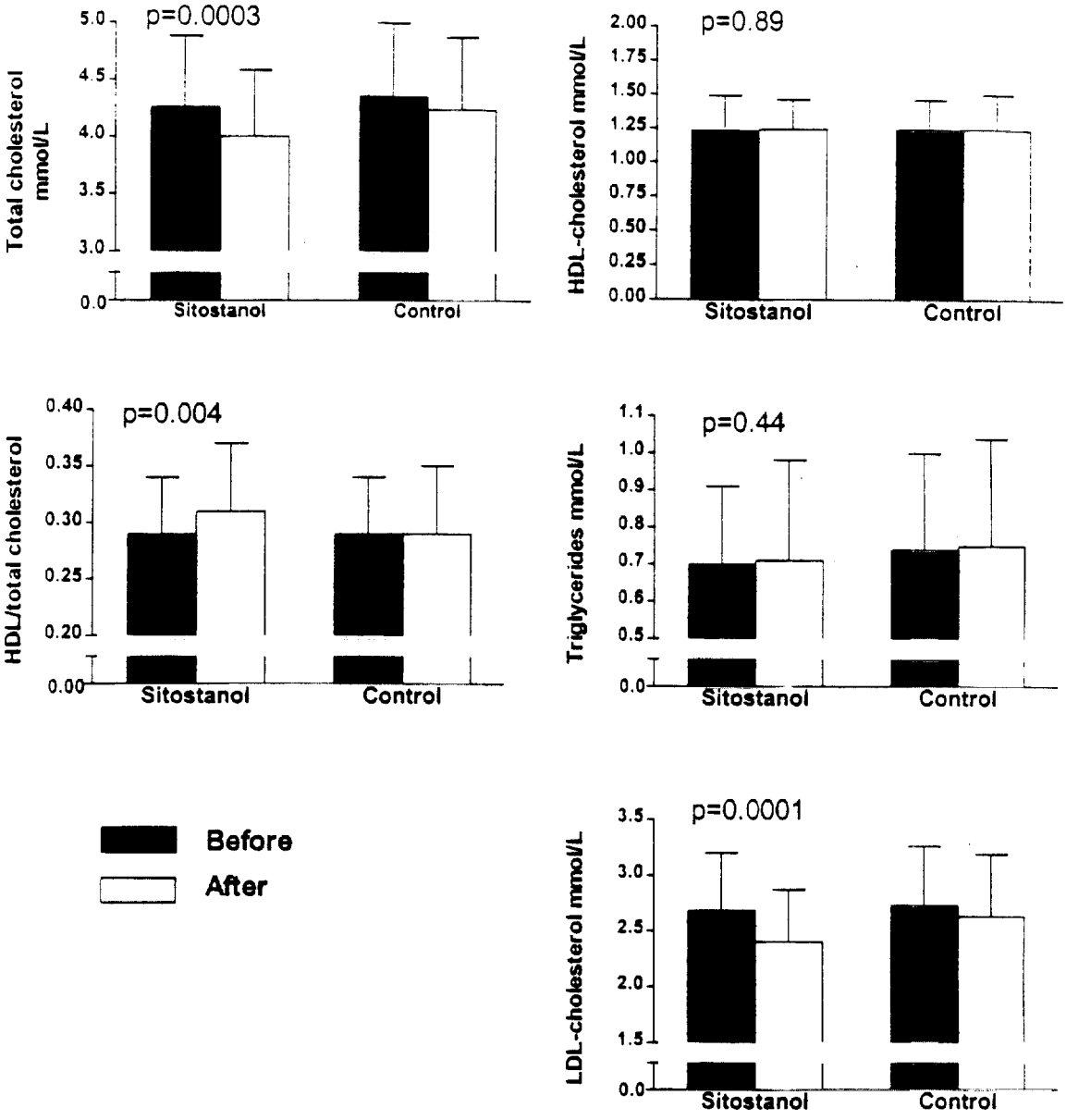


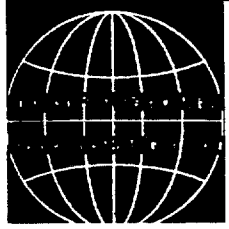
Table 1. Serum concentrations of α-tocopherol and β-carotene (mg/L) and their ratio to LDL (mg/mmol of cholesterol) before and after sitostanol and control study periods

	α-tocopherol		α-tocopherol/LDL		β-carotene		β-carotene/LDL	
	Sitostanol	Control	Sitostanol	Control	Sitostanol	Control	Sitostanol	Control
Before treatment	[illegible]	8.15±1.37	[illegible]	3.07±0.52	[illegible]	0.44±0.18	[illegible]	0.16±0.07
After treatment	[illegible]	8.25±1.34	[illegible]	3.18±0.58	[illegible]	0.45±0.23	[illegible]	0.17±0.09
Treatment effect	[illegible]	.	[illegible]	.	[illegible]	.	[illegible]	.
Treatment effect %	[illegible]	.	[illegible]	.	28.9	.	[illegible]	.
P-value	[illegible]	.	[illegible]	.	0.0001	.	[illegible]	.

Table 2. Serum concentrations of campesterol and Δ8-cholestenol (μg/dL) and their ratio to cholesterol (10^2 μmol/mmol of cholesterol) before and after sitostanol and control study periods

	Campesterol		Campesterol/chol		Δ8-cholestenol		Δ8-cholestenol/chol	
	Sitostanol	Control	Sitostanol	Control	Sitostanol	Control	Sitostanol	Control
Before treatment	[illegible]	598±205	[illegible]	393±122	[illegible]	14±7	[illegible]	9±5
After treatment	[illegible]	633±210	[illegible]	426±153	[illegible]	14±6	[illegible]	10±4
Treatment effect	[illegible]	.	[illegible]	.	[illegible]	.	[illegible]	.
Treatment effect %	[illegible]	.	[illegible]	.	[illegible]	.	[illegible]	.
P-value	0.0001	.	0.0001	.	[illegible]	.	0.002	.

PEDIATRIC RESEARCH



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Health Care for Urban Underserved Children

Roger E Spitzer, Marshall Denise, Arthea Brown, Amy I Spitzer, Earl Evans and Countess Medical Students and Volunteers, Pediatrics, SUNY Health Science Center, Syracuse, New York (Soon by:)

As a response to deteriorating infant mortality (20/1000 live births), low immunization rates (50%), and increasing use of the University Emergency Room for primary care, we developed a free pediatric clinic, including physician services, all medications, and immunizations, in the basement of one of the buildings of an urban low cost housing project, thereby eliminating costs and access as barriers to health care. House calls were also provided. Volunteers included medical students (at least 100/year), pediatricians, nurses and receptionists. During the 7 years of our existence, we reduced infant mortality in 1995 to 2 and 0 in 1996 and 1997, while the infant mortality for the rest of the city remained high at 14-20/1000 live births. Further, immunization rates rose from 50% to 90% in 1996 and 1997 while in the remainder of the city, rates persisted at 40-50%. Finally, we were able to promote a 40% reduction in ER use in 1997 compared to only a 10% reduction for patients other than those in the housing project. Although money and access were important, the community attributes this improvement to the humanistic and culturally sensitive care provided by our staff (especially our medical student volunteers). Equally important was the development of a community which felt responsibility for each other.

(Supported by the New York State Office of Minority Health)

773 *Poster Session IV, Tuesday, 5/4 (poster 51)*

Plant Stanol Ester Margarine as a Serum Cholesterol Lowering Agent in Healthy Children on a Diet Low in Saturated Fat and Cholesterol. The STRIP Project

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Atherosclerosis begins early in life and progresses slowly into symptomatic coronary heart disease in adulthood. Hypercholesterolemia is the main risk factor for atherosclerosis. Plant stanol margarine lowers serum cholesterol in mildly hypercholesterolemic adults and children with familial hypercholesterolemia.

To study the cholesterol lowering effect and tolerability of plant stanol margarine in healthy children we launched a study with double-blind cross-over design. The study subjects were advised to replace 20 g per day of their dietary fat with plant stanol or control margarine for three months. After six weeks' wash-out period the diets were switched over for three months. The study subjects (n=81) were six years old children taking part in the STRIP project (n=774) which is a randomized, prospective trial aimed at reducing the predisposition of young children to known atherosclerosis risk factors. All children belonged to the intervention group and, thus, were already on a diet low in saturated fat and cholesterol. They had normal or mildly elevated serum cholesterol values with mean cholesterol concentration of 166 mg/dl.

Results were analyzed according to intention-to-treat principle. Seventy-two children completed the study. Plant stanol margarine was well tolerated. The mean consumpuon of plant stanol margarine was 18.2 g per day, and the plant stanol dose was 1.49 g per day. Plant stanol margarine reduced serum total and LDL cholesterol concentration by 5.4 and 7.5 percent, respectively. Neither serum HDL cholesterol nor triglyceride concentration or safety markers (hematological markers, creatinine, lactate dehydrogenase, and glutamyl transferase) were affected by plant stanol margarine. Serum concentration of α-tocopherol remained unchanged during plant stanol treatment. Serum β-carotene/LDL cholesterol ratio decreased by 17.6 percent.

We conclude that plant stanol margarine lowers serum total and LDL cholesterol without adverse effects in healthy children who are already on a diet low in saturated fat and cholesterol.

774 *Poster Session IV, Tuesday, 5/4 (poster 3)*

Early Intervention for Children with Developmental Disorders: Who Do Physicians Refer?

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Parents' concerns about physician delay in referring their children for early intervention (E.I.) services led to a survey all of early interventionists in Ontario. Interventionists were polled regarding their perceptions of physician referrals to E.I. for children experiencing a wide variety of developmental disabilities. 44 of the 45 existing early intervention programs in Ontario responded to the survey, providing data on the 6116 children currently receiving an in home intervention service. 21% of the children were at environmental risk for delay, 9.5% had a primary communication or autistic spectrum disorder, 8% had diagnoses of specific genetic disorders, while 7% had an undiagnosed developmental delay. Other medical diagnoses, for example prematurity and neuromotor disorders, account for the remaining majority of the programs' caseloads. Optimizing child development and providing family support and counselling were the primary goals of the intervention. 33% of the total referrals to these programs were made by physicians and hospitals, while 23.5% were made directly by the parents. One third of the parent referrals, however, were made on the advice of their physician. Almost 60% of referrals from physicians were from pediatricians while 30% were from family physicians. Responses from 82% of the intervention programs indicate that physicians wait too long before making a referral for intervention, primarily because there has been no confirmed developmental diagnosis. The greatest delays in referral were for children with communication disorders and those at risk for delay due to environmental factors. All programs responded that when physicians do make referrals to intervention they are appropriate 90-100% of the time (i.e. high specificity). Over 50% of the intervention programs report that parents do not feel that physicians have time to appreciate their concerns about their child's development. Similarly they feel their concerns are trivialized through what is often false reassurance regarding normal outcome.

It appears that when physicians make referrals for early intervention, they are generally appropriate, however in spite of high specificity, there may be low sensitivity, particularly for children with developmental delay with unspecified etiology. A "wait and see" approach or delays in obtaining developmental consultation, due to long wait lists, may each be factors accounting for under or late referral. Parental anxiety and waste of critical periods of neuroplasticity are both reasons for physicians to increase sensitivity regarding the need for early developmental intervention.

775 *Health Services Research: Quality Platform Saturday, 5/1*

Measuring Quality of Care for Children and Adolescents in Managed Care

Joseph W Thompson, Department of Pediatrics, University of Arkansas College of Medicine, Little Rock, AR (Spon by: Richard F Jacobs)

Managed care systems increasingly dominate health care in commercial, medicaid, and the new State Children's Health Insurance Programs. However, concerns grow daily about quality of care provided to children and adolescents through these programs. Currently the industry standard for assessing quality are the performance indicators included within the Health Plan Employer Data and Information Set (HEDIS).

We examine the aspects of care for children and adolescents which are evaluated through performance measures in the current version of HEDIS. Results from analyses on data collected reflecting care to over 50 million Americans are reported. Finally, anticipated additions to the measurement set and strategies underway to resolve gaps in quality assessment are described.

HEDIS contains 15 standardized measures for monitoring quality of care for children and adolescents. These included 5 clinical measures (e.g., immunization rates), 5 utilization measures (e.g., myringotomy rates), and 5 access measures (e.g., well child visits). In 1997, commercial (n=339) and Medicaid (n=311) plans submitted data with most plans reporting clinical (84%) and utilization measures (90%), and fewest reporting access measures (31%).

Significant variation in health plan performance within markets is present for each measure of child and adolescent health care quality. Examples of variation in commercial plan performance in 1997 include: childhood immunization rate, mean=65.3%, sd=14.2; adolescent immunization rate, mean=53.8, sd=18.3; myringotomy rate, mean=22.4/1000, sd=12.4, children with recommended number of well-child visits by 15 months of age mean=55%, sd=25.1; adolescent well care visits annually, mean=32.3, sd=16.5. In addition, marked regional variation is observed for select measures and Medicaid plans frequently report poorer quality of care than their commercial counterparts. Preliminary evaluations of 1998 reported data suggest minimal health plan improvement.

In 1999, HEDIS will utilize the Consumer Assessment of Health Plan Survey. Comparative information from parents on experiences of care will be made publicly available including ratings of primary care providers, specialty care providers, and health plan quality. Efforts are underway to develop strategies for assessing quality of care provided to chronically ill children and those with disabilities.

Reported quality of care for children and adolescents varies dramatically across managed care systems in the United States. These differences have important clinical implications. The research community must engage to expand available measures for use, improve the quality of available data, and provide guidance in its interpretation.

776 *General Pediatrics III Platform, Tuesday, 5/4*

Bronchiolitis: A Four-Year Comparative Evaluation of the Impact of a Clinical Care Pathway on Patient Outcome and Resource Utilization Using a Large National Dataset

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It has been proposed that clinical care paths can favorably influence utilization and clinical outcomes by widely disseminating evidence-based, peer-recommended guidelines for patient care. Unique (causative) effects can be inferred if consistent intra-institutional effects on the targeted variables are documented over sequential years and if the test institution differs from other comparable institutions. We used the CHCA Pediatric Health Information System dataset to measure the impact of an evidence-based bronchiolitis care path at The Childrens Hospital, Denver (TCH) from 1994 (pre) to 1995-1997 (post) and to compare these changes to other children's hospitals. Our care path focused on clear admission and discharge criteria, individualized transition-anticipating orders, and "prove it or don't use it" criteria for the use of: RSV testing (not recommended for bed placement), bronchodilators, chest physiotherapy, and ribavirin. Overall, 10,636 bronchiolitis cases were studied comparing "days of use" for each utilization variable. Significant ($p<0.05$) results are shown in the table (↓= decreased, ↑ = increased).

Indicator	TCH Target	TCH 95-97 vs 94	TCH vs Others (96-97)
Admission Rate	↓	↓ (severity adj)	Comparable
Severity	↑	↑	Comparable
Length of Stay	↓	↓ (severity adj)	Comparable
Fatality Rate	0	0	↓
ICU Use	No Target	↓	↓
RSV Tests	↓	↓	↓
Bronchodilators	↓	Change in Use Pattern	Comparable
CPT	↓	↓	↓
Ribavirin	↓	↓	Comparable

Internally, TCH residents and attendings responded favorably to the bronchiolitis care path resulting in improved outcomes and decreased resource utilization. There were no fatalities and ICU days decreased even though the mean severity of admitted cases increased significantly. Targeted utilization was favorably affected, resulting in a cost savings of > $100,000 per year, whereas untargeted utilization was not. Nosocomial infections did not increase with decreased use of RSV testing. TCH differed favorably from other children's hospitals in several targeted categories. We conclude that large datasets can be useful in measuring compliance with evidence-based care guidelines which, in turn, can successfully influence utilization and evidence-predicted outcomes.

777 *Poster Session IV, Tuesday, 5/4 (poster 50)*

How Much Fat Is There in the Body Mass Index (BMI)?

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Although data from several sources suggests that the BMI of children is increasing in developed countries, we know of no data to verify that this increase is due to an increase in fat mass rather than lean body mass. Further, it is not clear that the same BMI standards for obesity can be applied to all ethnic groups because of possible differences in body composition. We therefore tested the hypothesis that lean body mass and fat mass are closely correlated to BMI in all groups of an ethnically diverse population and tested the implication of defining obesity based on percent body fat.

Methods: We measured height, weight and foot to foot bioelectrical impedance (BIA) of 2273 school children from 5 to 10.9 years old and body composition by dual energy x-ray absorptiometry (DEXA) in a subgroup of 63 children.

Results: DEXA and BIA techniques for evaluation of body composition gave closely correlated results for lean body mass (LBM) ($r=0.97$), fat mass ($r=0.98$) and percentage fat ($r=0.86$) in the subgroup of 63 children, validating foot to foot BIA to evaluate body composition in children. In 2273 children, using BIA results, we found close relationships between BMI and lean body mass ($r=0.68$), BMI and fat mass ($r=0.90$) and BMI and % fat ($r=0.75$) which was valid for all ethnic groups.

Using NHANES reference data BMI of >95th percentile as our definition of obesity, 14.3% of all children were obese. Age, gender and socioeconomic classification of the school area did not influence obesity rates. The percentage of obese children differed significantly ($p<0.0001$ by ANOVA) among the ethnic groups. Obesity rates varied considerably depending on which arbitrary definition of obesity was used, as shown in the table below as a percentage of children in each ethnic group fulfilling the specified definition of obesity.

	N	BMI > 95%	> 25% fat	> 30% fat	> 35% fat	> 40% fat
All children	2273	14.3	56.4	33.2	18.2	9.9
European	903	8.6	50.5	26.7	12.8	6.6
Maori	424	15.8	61.1	36.8	10.6	10.6
Pacific Island	627	24.1	64.3	41.8	24.1	14.2
Asian	180	5.6	36.7	17.8	6.7	2.8
Indian	93	11.8	71	49.5	34.4	20.4

Conclusions: 1.Childhood obesity is prevalent in 5-11 year olds in Auckland. 2. Obesity is established by the time of school entry at 5 years old. 3. Ethnic differences in obesity rates are clear and cannot be explained by differences in body composition. 4. Foot to foot BIA is a valid method for determining body composition in children. 5. The optimal definition of obesity is not clear and the implication of different definitions for public health planning is considerable.

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REFERENCE REMOVED

CONTAINED
CONFIDENTIAL
INFORMATION

Data Available Upon Request

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Evaluation of the use of β-sitostanol as a nonabsorbable marker for quantifying cholesterol absorption

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Summary For over a decade investigators have quantified cholesterol absorption by comparison of dietary intake and fecal excretion of isotopic cholesterol with that of β-sitosterol as a "nonabsorbable" marker. However, β-sitosterol might not be ideal due to its potential for absorption. We therefore carried out two studies to evaluate a new marker with less potential for absorption, [^{3}H]β-sitostanol. In the first study (Study I, n = 22), we compared absorption of [^{3}H]β-sitostanol and [^{14}C]β-sitosterol in a simultaneous dual-label continuous feeding ("phytosterol absorption") experiment. We observed a consistently higher ratio of [^{3}H]β-sitostanol/[^{14}C]β-sitosterol in the stool relative to diet on the first day of fecal collection (6.1% ± 3.2% loss of [^{3}H]β-sitosterol, range 3–12%), but thereafter, the ratio in stool was similar to that in diet. In Study II (n = 23), we compared cholesterol absorption directly using [^{3}H]β-sitosterol and [^{14}C]cholesterol, and, separately, [^{3}H]β-sitostanol and [^{14}C]cholesterol. We found that mean absorption between the two methods was similar (45% ± 11% versus 44% ± 10%, respectively, *P* difference = 0.40), and the two methods correlated well with one another (r = 0.83) when samples from all available days were used. Variability between the two methods was greater in individuals who absorbed more than 40% of cholesterol. Cholesterol loss on day 2 estimated from use of β-sitostanol as a nonabsorbable marker was predictive of absorption using ratios from days 4–6 (r = 0.80). These results suggest that, for the majority of subjects, β-sitosterol is a valid nonabsorbable marker for cholesterol absorption.–**Terry, J. G., B. L. McGill, and J. R. Crouse III.** Evaluation of the use of β-sitostanol as a nonabsorbable marker for quantifying cholesterol absorption. *J. Lipid Res.* 1995. **36:** 2267–2271.

Supplementary key words cholesterol • absorption • phytosterols

Cholesterol absorption has recently re-emerged as a potentially important contributor to the regulation of cholesterol metabolism (1, 2). Several methods have been advocated for quantifying absorption including the method of Zilversmit and Hughes (3, 4), the single dose isotopic diet/fecal ratio method (5), and the continuous feeding isotopic diet/fecal ratio method (6). The latter two methods depend on comparison of the excretion of labeled cholesterol with that of a labeled nonabsorbable marker, traditionally β-sitosterol.

Although poorly absorbed, it is recognized that about 5% of β-sitosterol is absorbed (7, 8), and in some patients up to 30% absorption can occur (9). β-Sitostanol, on the other hand, is thought to be nonabsorbable (10).

We therefore compared β-sitosterol and β-sitostanol as markers for cholesterol absorption in human beings.

METHODS

Subjects and diet

We carried out two studies of absorption in 45 healthy individuals. In the phytosterol absorption study (Study I) 22 volunteers were fed radiolabeled [^{14}C]β-sitosterol and [^{3}H]β-sitostanol daily for 6 days and stools were collected. Fecal ratio of isotopes was compared to that in the diet.

In the cholesterol absorption comparison study (Study II) 23 volunteers underwent two tests of cholesterol absorption separated by 1 month in which ^{14}C cholesterol and either [^{3}H]β-sitosterol or [^{3}H]β-sitostanol were dosed. For the two cholesterol absorption studies, 8 patients consumed ad lib diets at home and 15 consumed diets prepared in the Bowman Gray GCRC diet kitchen. Metabolic diets were consumed for 7–11 days and were eucaloric providing 33% of calories from fat, 48% from carbohydrate, and 19% from protein along with 300 mg cholesterol daily. Plasma concentrations of lipids and lipoproteins were measured in the CDC standardized Lipid Laboratory at Bowman Gray School of Medicine (11).

Absorption studies

[4-^{14}C]cholesterol (52 mCi/mmol) and custom synthesized [4-^{14}C]β-sitosterol (55 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, IL. [22,23-^{3}H]β-sitosterol (77 Ci/mmol) and [5,6-^{3}H]β-sitostanol (47 Ci/mmol) were custom synthesized by New England Nuclear, Boston, MA. Both tritiated β-sitosterol and β-sitostanol were produced from tritiated stigmasterol by sequential hydrogenation. Unlabeled β-sitosterol and stigmasterol were purchased form Sigma Chemical Co., St. Louis, MO. Five alpha-cholestane was purchased from Matreya, Inc., Pleasant Gap, PA. Unlabeled β-sitostanol was a gift from Dr. Margo Denke. Unlabeled β-sitostanol was > 90% pure by GLC (Hewlett-Packard 5890A) on a 15 M J&W DB17 column, and retention time was consistent with previous reports (12).

Isotopic purity of radiolabeled sterols was determined by HPLC (ISCO 2350) using a C-18 column (SGE) with isocratic buffer system (acetonitrile–isopropanol 1:1) and absorbance detection at 215 nm. All radiolabeled sterols were HPLC co-chromatographed with cold carriers and fractions were collected as previously described (13). Radioactivity was determined by liquid scintillation counting (Packard CA 2100 LSC). The lack

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Abbreviations: GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; BMI, body mass index; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein.

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of a double bond in β-sitostanol prevented absorbance detection at 215 nm, therefore, an additional step using GLC was required. For this, unlabeled β-sitostanol standard was run on HPLC and fractions were collected. The fractions were then subjected to GLC to determine the retention time of β-sitostanol in our HPLC system.

[^{14}C]cholesterol and [^{14}C]β-sitosterol were each > 98% isotopically pure. [^{3}H]β-sitosterol and [^{3}H]β-sitostanol were < 90% isotopically pure and required preparative purification by HPLC. Subsequent HPLC showed that > 96% of the activity from both [^{3}H]β-sitosterol and [^{3}H]β-sitostanol eluted under a single peak consistent with standards.

For Study I a mixture of 0.10 μCi[^{14}C]β-sitosterol and 0.31 μCi[^{3}H]βITC-NewBaskerville"-sitostanol was fed daily to ambulatory volunteers for 6 days (D1-6). Participants were instructed to collect one stool sample per day by the outpatient method of Hoffman, LaRusso, and Hoffman (14) either on D2-6 (n = 10) or only on D4-6 (n = 12).

Duplicate stool samples were saponified and the hexane-extracted neutral lipids were dried onto paper cones prior to combustion by Packard 306 Oxidizer as previously described (15). Samples of administered isotope were included as controls and frequent recovery checks were performed (recovery > 95%).

For quantification of β-sitosterol absorption the formula adopted was:

$$\beta\text{-sitosterol absorption} = 1 - \frac{\text{fecal } \beta\text{-sitosterol}/\beta\text{-sitostanol}}{\text{dietary } \beta\text{-sitosterol}/\beta\text{-sitostanol}}$$

This formula is a modification of one that has been shown to be reliable for quantifying cholesterol absorption in stool samples from D4-6; differential disappearance of isotope on D2 and D3 is characterized as isotope loss rather than absorption because loss could also occur through isotope exchange.

Study II participants underwent two studies of cholesterol absorption separated by 1 month in which they received either 0.11 μCi [^{14}C]cholesterol and 0.29 μCi [^{3}H]β-sitosterol or alternatively 0.10 μCi [^{14}C]cholesterol and 0.29 μCi [^{3}H]β-sitostanol for 6 consecutive days. Subjects were assigned at random to their sequence of studies and the sample analyst was blinded to the order of assignment.

Daily loss of isotope as well as means for D4-6 were used to determine β-sitosterol absorption or cholesterol absorption as previously described (6). Means and standard deviations and associations were compared between periods and groups using paired *t*-tests and correlation statistics.

RESULTS

Study I

Participants in Study I were 67 ± 6 years of age (range 49-75) and 50% female; they had body mass index (BMI) of 27 ± 4 (range 22-38). They were slightly hypercholesterolemic overall with plasma concentration of total cholesterol (TC) of 236 ± 31 mg/dl (range 186-298 mg/dl) and low density lipoprotein cholesterol (LDL) of 154 ± 22 mg/dl (range 122-203 mg/dl). Plasma concentration of triglycerides (TG) for Study I participants was 218 ± 161 mg/dl (range 69-835 mg/dl).

Twenty-two participants completed Study I that compared [^{14}C]β-sitosterol and [^{3}H]β-sitostanol absorption (**Fig. 1**). All participants were asked to collect stool samples on D4-6 of Study I while a subset of participants (n = 10) was requested to collect samples on D2 and D3 as well. Mean % absorption of β-sitosterol on D4, D5, and D6 was 2.5% ± 3.9%, -1.3% ± 3.6%, and -1.8%, ± 4.6% respectively (mean % absorption D4-6 = -0.1% ± 2.7%, and did not differ from 0% absorption). In those subjects who submitted samples from D2 and D3, preferential loss of β-sitosterol from stool on day 1 was reflected in a ratio of fecal/diet [^{3}H]β-sitostanol/[^{14}C]β-sitosterol that was consistently > 1.0 in stool from D2 (per cent loss [^{14}C]β-sitosterol on D2 = 6.1% ± 3.2%, range 3% to 12.5%). On D3 per cent loss of [^{14}C]β-sitosterol was 0.3% ± 7.0%.

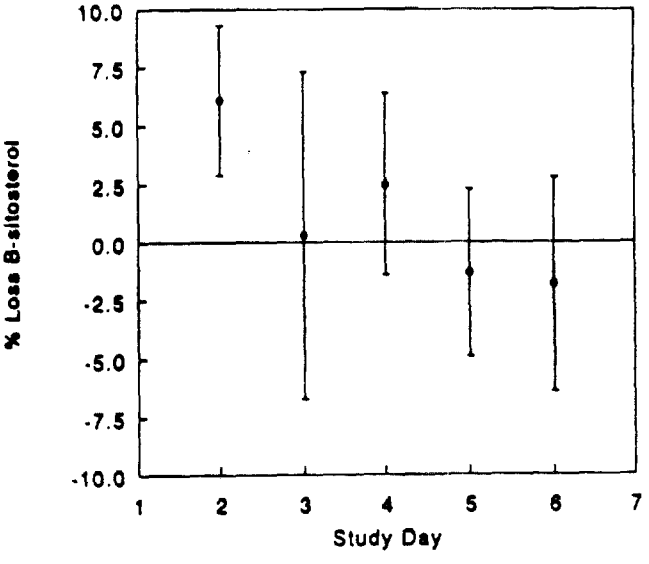

Fig. 1. Mean ± standard deviation percent loss of β-sitosterol relative to β-sitostanol for participants on days 2-6 of phytosterol absorption study (Study I). Days 4-6, n = 23; days 2-3, n = 10. Positive "% loss" indicates greater loss of β-sitosterol than β-sitostanol.

Study II

Study II participants were 65 ± 8 years of age (range 35–73) and were 52% female; they had BMI of 27 ± 4 (range 20–38). These subjects had overall lipids similar to the previous group with TC 226 ± 39 mg/dl (range 159–290 mg/dl), LDLC 151 ± 32 mg/dl (range 85–198 mg/dl), and TG 179 ± 96 mg/dl (range 76–506 mg/dl).

In Study II, cholesterol absorption was compared in 23 individuals using [^{3}H]β-sitostero l, and, on a different occasion, [^{3}H]β-sitostanol as nonabsorbable markers for ^{14}C cholesterol absorption. Comparisons are presented in **Table 1** and the correlation between the two estimates of absorption is illustrated in **Fig. 2.** Overall, there was no consistent difference in absorption as measured by the two methods. Variability was greater in individuals who absorbed more than 40% of cholesterol, but even in these patients, cholesterol absorption measured by the two methods was similar. Three of 23 subjects showed a statistically significant difference in cholesterol absorption using β-sitosterol compared to β-sitostanol (subjects number 8, 9, and 15). There was no consistent pattern to these differences. Overall, the correlation between the two cholesterol absorption methods on D4–6 was $r = 0.76$, $P < 0.0001$. When results from all available days were used (D2–6), the correlation was $r = 0.83$, $P < 0.0001$. Cholesterol loss on D2 as estimated from use of β-sitostanol as a nonabsorbable marker was predictive of absorption using isotope ratios of β-sitosterol and cholesterol from D4–6 ($r = 0.80$, $P < 0.0001$, n = 17). D2 cholesterol loss estimated from use of β-sitosterol as a nonabsorbable marker correlated less well with the published method ($r = 0.56$, $P < 0.02$, n = 18) as previously suggested (6).

Availability of two highly correlated indices of cholesterol absorption allowed us to derive a stable estimate of cholesterol absorption from the 23 individuals. No association was observed between per cent cholesterol absorption and age, or plasma TC or LDLC; statistically significant (negative) associations were found between cholesterol absorption and BMI ($r = -0.46$, $P < 0.03$) and plasma TG ($r = -0.60$, $P < 0.003$).

TABLE 1. Percent cholesterol absorption (mean ± SD) by phytosterol marker for individual participants in cholesterol absorption comparison (Study II)

Subject	β-Sitosterol	β-Sitostanol	*P* Value[a]
1	37±5	36±9	0.932
2	34±4	25±4	0.085
3	52±8	59±9	0.439
4	36±2	31±7	0.425
5	24±6	27±5	0.574
6	42±10	41±9	0.924
7	45±3	42±2	0.317
8	45±1	56±5	0.046
9	52±1	36±5	0.039
10	58±4	43±2	0.069
11	59±3	62±1	0.159
12	40±1	44±4	0.165
13	59±7	60±3	0.808
14	46±2	46±2	0.999
15	72±3	54±3	0.014
16	45±6	44±3	0.844
17	58±9	59±8	0.910
18	36±2	42±4	0.328
19	38±1	41±1	0.732
20	36±8	43±5	0.457
21	34±5	35±13	0.925
22	42±3	37±5	0.259
23	43±2	41±2	0.944
All subjects	45±11	44±10	0.404

[a]Significance level by Student's *t*-test.

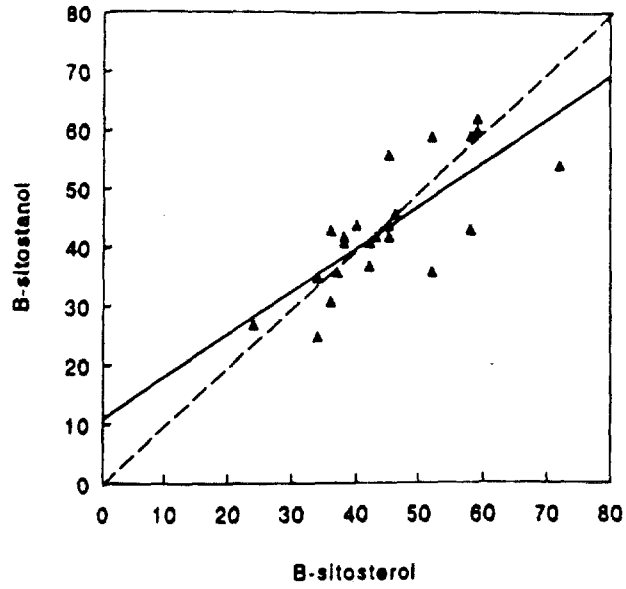

Fig. 2. Percent cholesterol absorption (days 4–6) measured twice in the same 23 individuals using β-sitosterol compared to β-sitostanol as nonabsorbable marker. Solid line, best fit line (equation: $y = 0.729x + 10.832$); dashed line, line of identity.

DISCUSSION

Several methods have been proposed for measuring cholesterol absorption. The most commonly used of these at present include the Zilversmit method (3) that involves simultaneous intravenous and oral administration of radiolabeled cholesterol (4), the fecal isotope ratio method that involves single dosing of individuals with radiolabeled cholesterol and β-sitosterol with complete fecal collections for 7–10 days thereafter (5), and the continuous oral isotope feeding ratio method that involves continuous dosing over 7 days with radiolabeled cholesterol and β-sitosterol and collection of casual aliquots of stool for analysis (6).

The advantage of the Zilversmit method (3) is its ease of accomplishment, requiring only sampling of plasma after administration of isotope for quantification of cholesterol absorption. The disadvantage of the method, as well as of the single dose ratio method, is that it measures only the cholesterol absorption of the meal with which it is fed. The only method that permits quantification of cholesterol absorption over several days and that provides a mean and standard deviation for cholesterol absorption over that period of time is the continuous isotope feeding method. This method also has an advantage over the single dose isotope ratio method in that casual (not complete) stool collections adequately quantify cholesterol absorption.

A theoretical limitation of the methods using radiolabeled β-sitosterol is the potential for its absorption. Little is known about the variability of β-sitosterol absorption in the population. Approximately 5% of β-sitosterol is thought to be absorbed (7, 8), but certain individuals have been identified who absorb up to 30% of the β-sitosterol in their diets (9). Wide variability among individuals in absorption of β-sitosterol might theoretically prevent accurate measurement of cholesterol absorption. Because only 40–60% of cholesterol is absorbed, small differences in absorption of the "nonabsorbable" marker could lead to larger differences in apparent cholesterol absorption. To the extent that β-sitosterol is absorbed, apparent absorption of cholesterol would be less than "true" percent absorption.

β-Sitostanol is recognized as a nonabsorbable phytosterol (10) and has been used as a nonabsorbable marker in one study in which stable isotopes were fed and fecal collections were obtained (16). The latter method requires isolation and analysis of individual sterols and their metabolites from stool. Use of radiolabeled β-sitostanol as a nonabsorbable marker for cholesterol absorption is attractive as *1)* it is not absorbed; and *2)* complex analysis of stool is not necessary for its quantification.

Accordingly, these studies were initiated to compare β-sitosterol and β-sitostanol with one another for their utility as nonabsorbable markers for cholesterol absorption. In Study I we observed a differential loss of β-sitosterol from the stool compared to β-sitostanol on the first day and extending in some patients to the second day of administration. By the 3rd to 4th day, the isotope ratio in the stool was very similar to that fed, suggesting that in most patients β-sitosterol should be a valid nonabsorbable marker for cholesterol absorption after at least 4 days of isotope feeding. The mechanism for the differential loss of β-sitosterol on day 1 is unclear. There are two possibilities. First, β-sitosterol may exchange with sterol in mucosal cells to a different extent than β-sitostanol and may be lost from the lumen through this mechanism and not appear in the stool until the second or third day of β-sitosterol feeding. Second, β-sitosterol may be absorbed and resecreted very rapidly. The latter is possible in view of the relatively rapid turnover of intravenously administered β-sitosterol as described by Salen, Ahrens, and Grundy (7).

When we quantified absorption of cholesterol through use of β-sitosterol, and, separately, β-sitostanol as nonabsorbable markers, we could show no marked differences between the two isotopes. Although for most subjects cholesterol absorption estimated by the two markers was comparable, eleven individuals had higher apparent absorption with β-sitostanol as the nonabsorbable marker (statistically significant in one individual) and five individuals had higher apparent absorption with β-sitosterol (statistically significant in two

individuals). No consistent pattern of difference was observed between cholesterol absorption measured by either nonabsorbable marker when comparing results from days 4–6. Overall, the difference in cholesterol absorption measured by β-sitosterol and β-sitostanol (mean %-difference 0.4% ± 21.1%) in the present study was similar to that expected for absorption studies separated by 1 month using the same nonabsorbable isotopic marker (2.8% ± 14.2% using [^{3}H]β-sitosterol) (15).

Despite their similar overall results for cholesterol absorption, apparent differences exist between β-sitosterol and β-sitostanol. Cholesterol absorption estimated by isotopic β-sitostanol on day 2 was more reflective of that measured on days 4–6 than was cholesterol absorption as estimated by isotopic β-sitosterol itself on day 2 (r = 0.80 versus 0.56, respectively). This observation, along with the findings of Study I, confirms the notion that isotopic β-sitosterol is only valid as a cholesterol absorption marker after several days of feeding to allow equilibrium in the intestine (6).

In summary, these data support the continued use of β-sitosterol as a nonabsorbable marker for cholesterol absorption. On the other hand it is evident that there are differences in the metabolism of β-sitosterol and β-sitostanol, particularly on the first day of feeding, and uncommon individual patients have previously been shown to absorb β-sitosterol (9). For this reason, we believe that it is also valid to use β-sitostanol as a nonabsorbable marker for cholesterol absorption ■

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Genotoxicity Evaluation of Wood-Derived and Vegetable Oil-Derived Stanol Esters

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Plant stanol esters from wood and vegetable oil sources were tested for genotoxicity in bacterial (*Salmonella typhimurium*) and mammalian cell (L5178Y) gene mutation assays and in a mammalian cell chromosome aberration assay (CHO cells). The two stanol ester formulations were tested separately at doses up to the limit of solubility, with and without the addition of an Aroclor-induced rat liver microsome metabolic activation system (S9 mix). All tests were performed in duplicate and gave negative results for both wood and vegetable oil stanol ester formulations. Thus, plant stanol esters are not genotoxic under the conditions of exposure tested. © 1999 Academic Press

INTRODUCTION

Plant stanol esters are fatty acid esters of plant stanols which are prepared by hydrogenation from naturally occurring plant sterols found in oil extracted from wood (tall oil) and various vegetable oils. For the commercial product, plant stanol esters are prepared by interesterification of plant stanols with the fatty acids such as canola oil to improve their solubility in fats. The plant stanols themselves are prepared by hydrogenation of mixed plant sterols derived from wood or vegetable oil. The conversion of the plant sterols to the plant stanols reduces their gastrointestinal absorption to negligible levels and increases their effectiveness at inhibiting cholesterol absorption (Heinemann *et al.*, 1991; Sugano *et al.*, 1977).

Plant stanol fatty acid ester mixtures derived from wood (tall oil) or vegetable oil have been used in Finland for some years in margarine spread produced by the Raisio Group to help reduce serum total cholesterol and LDL cholesterol levels (Miettinen *et al.*, 1995). The rationale for this use of hydrogenated plant sterol products is that they can contribute to maintenance of cardiovascular health by reducing cholesterol absorption from the diet and from bile without themselves being absorbed or having any undesirable pharmacological or toxicological effect.

The principal plant stanol esters are mixed fatty acid esters of the 5α-plant stanols, sitostanol and campestanol. Sitostanol (24-ethylcholestan-3-ol, CAS No. 19466-47-8) is formed by the hydrogenation of the Δ^5-mono-unsaturated plant sterol, sitosterol (24-ethylcholest-5-en-3β-ol, CAS No. 83-46-5), and also by the complete hydrogenation of the $\Delta^{5,22}$-di-unsaturated plant sterol, stigmasterol (24-ethylcholest-5,22-dien-3β-ol, CAS No. 83-48-7), hence the alternative name "stigmastanol." Campestanol (24-methylcholestan-3β-ol) is formed by the hydrogenation of the Δ^5-mono-unsaturated plant sterol, campesterol (24-methylcholest-5-en-3β-ol, CAS No. 474-62-4).

The structures of sitostanol, of its immediate precursors sitosterol and stigmasterol, of campestanol, and of its immediate precursor campesterol are shown in Fig. 1. (The structure of cholesterol, which differs from campesterol and sitosterol by the presence of a single methyl or ethyl group, respectively, is shown for comparison.)

As part of a comprehensive safety assessment program, the genotoxic potential of plant stanol esters derived from wood and from vegetable oil were tested in a battery consisting of an Ames assay in *Salmonella typhimurium* bacteria, a mammalian cell gene mutation assay in L5178Y mouse lymphoma cells, and a mammalian cell chromosome aberration assay in Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

Samples of wood-derived and vegetable oil-derived plant stanol fatty acid esters, grayish-white waxy solids, were provided by Raision Tehtaat OY AB, Raisio, Finland. In each assay, wood-derived plant stanol fatty acid esters and vegetable oil-derived plant stanol fatty acid esters were tested separately.

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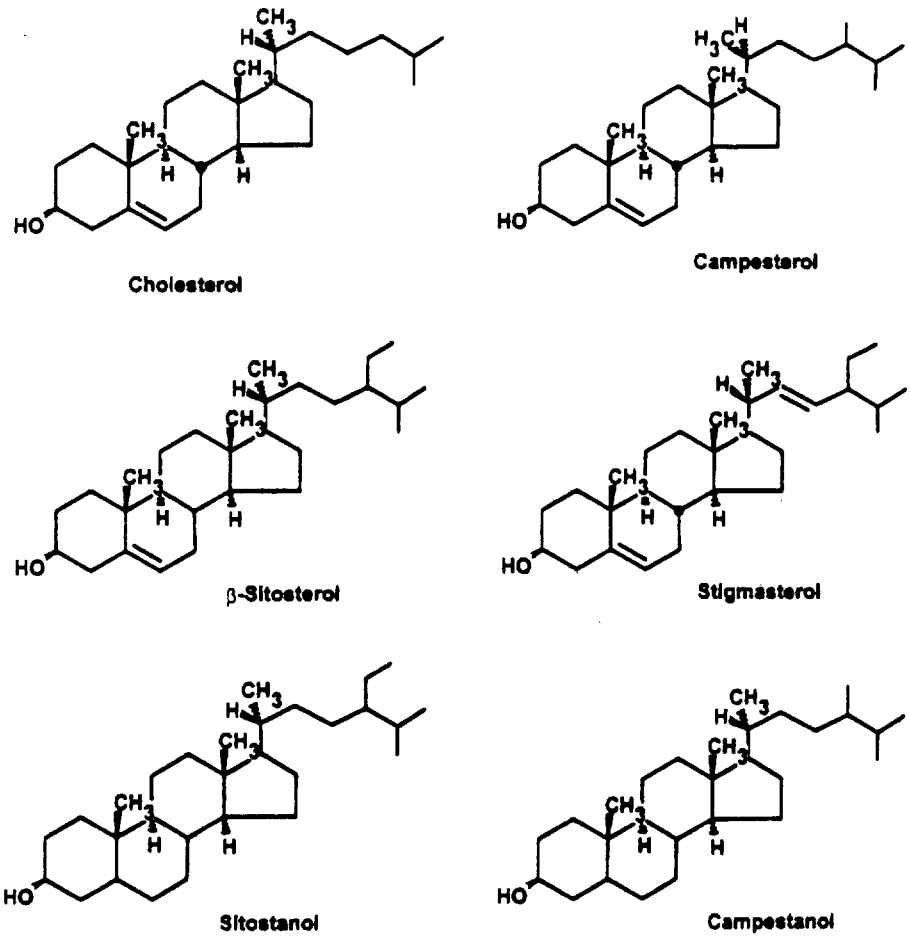

FIG. 1. Structure of cholesterol and some plant sterols.

Ames Assay

The assay was performed using *S. typhimurium* strains TA 98, TA 100, TA 1535, and TA 1537 following the methods of Ames *et al.* (1975) and Maron and Ames (1983), in compliance with OECD guideline 471 and the B.14 guideline of the EEC. Metabolic activation was provided by an Aroclor-induced rat liver microsome fraction (S9 mix). For each of the wood-derived and vegetable oil-derived plant stanol fatty acid esters, five different concentrations of stanol ester were tested, up to the limit of solubility (5000 μg/plate).

L5178Y TK$^{+/-}$ Assay

The assay was performed in the presence and absence of an Aroclor-induced rat liver microsome metabolic activation system (S9 mix), in compliance with EPA Health Effects Testing Guidelines, OECD Guideline 476, and the EEC protocol, "Gene Mutation Test—Mammalian Cells *in Vitro*" of Council Directive 87/302/EEC. The assay system detects forward mutations (basepair mutations, frameshift mutations, and small deletions) affecting the thymidine kinase (TK) locus by selecting for cells resistant to the thymidine analog trifluorothymidine. L5178Y cells (L5178Y tk$^{+/-}$ 3.2.7.c line) were obtained from Dr. J. Cole, MRC Cell Mutation Unit, University of Sussex, United Kingdom. The cells were stored as frozen stock cultures in liquid nitrogen and grown in RPMI 1640 medium, with Hepes and Glutamax-1, supplemented with heat-inactivated horse serum [10% (v/v) for culture in flasks; 20% for growing in microtiter plates] and penicillin/streptomycin. Each new stock culture was checked for mycoplasma contamination (absent) and karyotype stability (stable). For testing, fresh cultures were seeded at about 10^7 cells/75-cm^2 culture flask in 50 ml of medium and grown for 5 to 7 days prior to treatment. On the day of treatment, growth rate (11 ± 2 h) and viability (>90% by trypan blue exclusion) were tested.

Wood-derived and vegetable oil-derived stanol esters were tested separately with and without S9 at concentrations of 20–500 μg/ml and 250–3000 μg/ml, respectively. The highest test dose was limited by the solu-

TABLE 1

***Salmonella*/Microsome Mutagenicity Test with Wood-Derived and Vegetable Oil-Derived Stanol Esters [Mutants/Plate; Mean of Three Plates (sd)]**

Dose (μg/plate)	TA 1535		TA 1537		TA 90		TA 100	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
				Wood-derived stanol ester				
0	17 (4)	14 (3)	13 (2)	13 (5)	28 (5)	40 (2)	131 (9)	133 (2)
62	18 (4)	17 (2)	10 (2)	12 (1)	24 (2)	48 (2)	139 (14)	127 (7)
185	16 (6)	17 (3)	9 (2)	13 (2)	29 (3)	47 (9)	135 (16)	137 (20)
556	18 (3)	15 (4)	9 (3)	19 (0)	24 (5)	42 (2)	122 (7)	145 (7)
1667	15 (2)	15 (4)	8 (3)	10 (2)	20 (6)	40 (7)	133 (10)	134 (3)
5000	15 (3)	19 (5)	11 (5)	14 (1)	30 (8)	45 (3)	131 (14)	141 (4)
Positive control[a]	543 (21)	710 (56)	1464 (269)	261 (16)	1111 (18)	1476 (50)	616 (19)	1723 (55)
				Vegetable oil-derived stanol ester				
0	23 (4)	16 (6)	14 (6)	8 (1)	23 (4)	45 (5)	143 (13)	147 (9)
62	20 (0)	21 (2)	13 (3)	11 (3)	28 (13)	51 (2)	152 (1)	165 (6)
185	19 (2)	19 (0)	14 (1)	15 (3)	30 (9)	43 (8)	157 (8)	129 (20)
556	26 (3)	20 (8)	13 (4)	12 (4)	33 (8)	41 (10)	151 (12)	160 (13)
1667	23 (6)	22 (10)	9 (4)	19 (6)	22 (4)	49 (5)	146 (11)	141 (9)
5000	50 (21)	25 (10)	19 (4)	20 (5)	30 (6)	49 (6)	127 (8)	137 (11)
Positive control[a]	501 (48)	642 (22)	1193 (283)	212 (12)	971 (164)	940 (44)	662 (52)	1674 (180)

Note. sd, standard deviation.

[a] In the absence of S9, the positive controls were sodium azide (1 μg/plate) with TA 1535 and TA 100; 9-aminoacridine (80 μg/plate) with TA 1537; and 2-nitrofluorene (2 μg/plate) with TA 98. With S9, they were 2-aminoanthracene (2 μg/plate) with TA 1535, TA 98, and TA 100; and benzo(*a*)pyrene (4 μg/plate) with TA 1537.

bility of the stanol esters in the culture medium. At concentrations above 500 μg/ml, the vegetable oil-derived stanol esters were not completely dissolved in the final medium at the end of treatment, indicating that 500 μg/ml approximated the lowest insoluble concentration. The stanol esters were suspended in DMSO at 60°C (with sonication) at 500 mg/ml or less and further diluted in DMSO prior to addition to the culture medium (final DMSO concentration, 1%). Exposure was for 4 h at 37°C. Methyl methane-sulfonate (MMS, 0.2 mM) was used as a positive control without S9, and 3-methylcholanthrene (MCA, 10 μg/ml) was used as a positive control with S9. At the end of the treatment period, the cells were washed with medium and aliquots were cultured (with 20% horse serum) at 10 cells/ml to determine the initial cloning efficiency. The remaining cells were cultured for 44 h at 37°C to allow phenotypic expression of induced mutants. After 20 h, the cells were counted and diluted, if necessary, to 200,000 cells/ml in culture medium. At the end of the expression period, aliquots were counted and diluted to 10 cells/ml and transferred to 96-well microtiter plates (200 μl/well), and cloning efficiency was determined using the zero term of the Poisson distribution (Cole *et al.*, 1983). The remaining cells were diluted to 10,000 cells/ml in cloning medium (with 20% horse serum) containing trifluorothymidine (TFT) at 4 μg/ml to select for mutants, and 200-μl aliquots were transferred to each of two 96-well microtiter plates and incubated at 37°C for 10–14 days. The cloning efficiency in TFT was also calculated using the zero term of the Poisson distribution, and the mutant frequency per 1,000,000 clonable cells was calculated (Cole *et al.*, 1983).

CHO Cell Chromosome Aberration Assay

The assay was performed in the presence and absence of an Aroclor-induced rat liver microsome metabolic activation system (S9 mix), in compliance with OECD Guideline 473 (Genetic Toxicology: *In Vitro* Mammalian Cytogenetic Test), and the B.10 guideline of EEC Council Directive 67/548, Seventeenth Amendment, Part B. CHO K-1 cells, obtained from Professor A. T. Natarajan, University of Leiden, The Netherlands, were stored as frozen stock cultures (passage 13) in liquid nitrogen and grown in Ham's F-12 culture medium with Glutamax-1, supplemented with heat-inactivated fetal calf serum (10%), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells were cultured for 24 h prior to treatment with the test compounds.

Stanol esters were separately tested with and without S9 at concentrations of 125–500 μg/ml (wood-derived stanol esters) and 500–2000 μg/ml (vegetable oil-derived stanol esters). The highest test dose for each of the stanol ester formulations was limited by the solubility of the stanol esters in the culture medium. The stanol esters

TABLE 2

L5178Y Mouse Lymphoma Mutagenicity Test with Wood-Derived and Vegetable Oil-Derived Stanol Esters

Dose (μg/ml)	Initial cloning efficiency		Final cloning efficiency		Mutant cloning efficiency ×10⁶		Mutants per 10⁶ clonable cells		Large mutant colonies (%)	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
					Wood-derived stanol ester					
0[a]	0.91	0.91	1.06	0.87	88	117	83	135	71	53
20	0.74	0.81	0.88	1.02	120	147	136	145	—	—
80	0.98	0.96	0.90	0.90	79	162	88	180	—	—
160	0.75	0.84	1.00	1.11	117	120	117	109	—	—
320	0.85	0.84	0.90	1.06	130	137	145	129	—	—
400	0.82	0.81	0.74	0.81	127	172	172	213	42	45
500	0.96	0.85	1.13	0.88	70	114	62	129	60	46
Positive control[b]	0.53	0.65	0.41	0.59	180	432	435	731	24	46
					Vegetable oil-derived stanol ester					
0[a]	0.81/0.81	0.91/0.77	0.73/0.93	0.82/0.82	61/46	61/82	84/50	74/99	17/50	44/28
250	0.87	0.79	0.95	0.85	41	85	43	100	—	—
500	0.76	0.72	0.77	0.76	49	88	64	116	—	—
1000	0.81	0.84	0.95	0.91	55	107	58	117	—	—
2000	0.85	0.81	1.08	1.02	67	82	62	80	—	—
3000	0.70	0.67	1.06	0.96	46	94	44	98	—	—
Positive control[b]	0.42	0.53	0.40	0.61	130	302	329	497	30	31

[a] Results are shown for the solvent control (1% DMSO; culture medium control gave similar results, not shown). Duplicate solvent control cultures were used with vegetable oil-derived stanol ester; results are shown for both, separated by /.

[b] Methyl methanesulfonate (0.2 mM) without S9; 3-methylcholanthrene (10 μg/ml) with S9.

were suspended in DMSO at 62°C (with sonication) at 500 mg/ml or less and further diluted in DMSO prior to addition to the culture medium (final DMSO concentration, 1%). Exposure to the test substances was for 18 or 32 h at 37°C without S9 and 3 h with S9. In both cases, cells were harvested 18 and 32 h after the start of treatment. Mytomycin C (0.025 μg/ml) was used as a positive control without S9, and cyclophosphamide (3.75 μg/ml) was used as a positive control with S9 (harvest was at 18 h only with the positive control substances). Two hours before harvest time, colcemid (0.1 μg/ml) was added to arrest cells in metaphase. The cells were then harvested by trypsinization, treated for 15 min at 37°C with hypotonic solution (1% sodium citrate), fixed with 3:1 methanol:acetic acid, transferred to clean glass slides (two per culture), stained with 2% Giemsa, rinsed, dried, embedded with a Tissue-TEK coverslip, coded, and scored "blind." At least 1000 nuclei from each culture (500/slide) were examined to determine the mitotic index. For each treatment, 200 well-spread metaphases were analyzed for aberrations.

RESULTS

Ames Assay

Neither of the plant stanol ester preparations was toxic to the cells at any concentration tested, with or without metabolic activation, as indicated by the absence of a decrease in the number of mutants per plate. At the highest dose (5000 μg/plate), however, a precipitate was seen in all plates indicating the limit of test substance solubility. Table 1 shows the results of one of the two duplicate assays performed with each stanol ester preparation (the second assay gave similar results, not shown). No evidence of a significant, dose-related increase in mutant frequency was seen with either plant stanol ester preparation in any strain, with or without metabolic activation. The positive control substances gave the expected increase in revertant number [in one trial, a less than expected number of revertants was seen in strain TA 1537 with the positive control substance (benzo(*a*)pyrene) with S9; this portion of the trial was repeated and the expected response was seen with the positive control, but no increase in mutant number was seen with the test substance, data not shown].

L5178Y TK$^{+/-}$ Assay

Neither of the stanol ester preparations was toxic to the cells at any concentration tested, with or without metabolic activation, as indicated by the absence of a reduction in the relative initial cell yield and cloning efficiency. At the highest concentrations of wood-derived (500 μg/ml) and vegetable oil-derived (3000 μg/