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An Escalating Dose Oral Gavage Study of 3β-Acetoxyandrost-5-ene-7,17-dione (7-oxo-DHEA-acetate) in Rhesus Monkeys

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To test the effects of 7-oxo-dehydroepiandrosterone-3 acetate (hereafter 7-ODA) in Rhesus macaques the steroid was administered by oral gavage to two male and two female monkeys. Dose levels of 250, 500, and 1,000 mg/kg body weight (BW)/day were administered on days 1, 3, and 5 respectively, and 1,000 mg/kg on days 7 through 11. Each group received the dose in a volume of 10 ml/kg BW. All animals survived to the scheduled sacrifice on day 12. No adverse clinical effects of 7-ODA were observed at the 250 or 500 mg/kg doses. Females vomited on non-treatment days and all animals vomited on some days after being given the 1000 mg/kg dose. Excessive salivation was observed before or immediately after dosing on days 9 through 11. Appearance, behavior and body weights were not altered by the treatments. Visual examination of all body cavities, and macroscopic and microscopic examination of 42 different organs and tissues found no lesions or abnormalities.

The diverse metabolic effects of DHEA have been briefly described in the accompanying paper (1). The many positive, potentially useful properties of this steroid are partially negated by the hazards it could introduce to some individuals as a result of it's being converted to either male or female sex hormones in greater than normal amounts. The most consistently-reported adverse effects seen in humans have been hirsutism and acne (2–4). A more suitable steroid for therapeutic purposes is 7-oxo-DHEA for it is more potent than DHEA in many of the desired functions, is not recognized by the androgen receptor in prostatic tissue (5), and cannot be converted to either testosterone (6) or estrogens (7). To prepare for possible use of this naturally-occurring steroid in humans we tested the tolerance of a primate species for 7-ODA. We have previously established that this acetyl ester is readily hydrolyzed in animals and is as effective as equimolar amounts of the free steroid (8). In the present study we find that 7-ODA in doses up to 500 mg/kg body weight were well tolerated and caused no significant changes in organ structure or blood composition. Such doses far exceed those that might be given to humans.

METHODS AND MATERIALS

Methods. This study was designed in accordance with the United States Food and Drug Administration's Good Laboratory Practice Regulations for Nonclinical Laboratory Studies, 21CFR 58, with the exception of the dose analysis. The study was conducted at Covance Laboratories Inc., Madison, Wisconsin (protocol TP 5301).

Materials. All materials and preparative procedures were as described in the accompanying paper (1).

Animals. Three male and three female rhesus macaques (HRP, Inc., Alice, Texas) were acclimated for at least 30 days before initiation of treatment. During acclimation the animals were examined for abnormalities, given three tuberculosis tests, a physical examination, and a fecal flotation test for parasites. They were housed at a temperature of 19° to 26°C, a relative humidity level of 50% ± 20%, and a 12-hour light/12 hour dark cycle. The animals were maintained individually in stainless steel cages. Their weights varied from 3.3 to 4.1 kg at initiation of treatment. Each animal was assigned a permanent number upon arrival and identified with an ear tag. All data for each animal were recorded under this number.

The monkeys were fed Certified Primate Diet #5048 (PMI Feeds, Inc., Richmond, Indiana) ad libitum. The diet was supplemented with bananas, apples and cereal (not quantified). Water was provided ad libitum; its quality was described (1).

Treatment. Two male and two female macaques received the test material 7-ODA by oral gavage at dose levels of 250, 500, and 1,000 mg/kg BW on days 1, 3 and 5 respectively. Dose concentrations were prepared as described in the accompanying paper. The oral gavage route was chosen because the intended route of administration to humans is orally. Because no clear evidence of toxicity was observed after a single dose of 1,000 mg/ kg, the animals were treated with 1,000 mg/kg for 5 consecutive days (days 7 through 11). All dose

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Abbreviations used: DHEA, dehydroepiandrosterone; 7-ODA or 7-oxo-DHEA, 3β-acetoxyandrost-5-ene-7,17-dione; MC, methylcellulose; Tween 80, polyoxyethylene sorbitan monooleate.

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TABLE 1

Blood Constituents Measured

Hematology	
Red blood cell count	Differential blood cell count
Hemoglobin	Reticulocyte count
Hematocrit	Corrected white cell count
Mean corpuscular volume	Segmented neutrophil count
Mean corpuscular hemoglobin	Band neutrophil count
Platelet count	Lymphocyte count
Prothrombin time	Monocyte count
Blood cell morphology	Eosinophil count
White blood cell count	Basophil count

Clinical Chemistry	
Calcium	Triglycerides
Chloride	Total protein
Inorganic phosphorus	Albumin
Potassium	Globulin
Sodium	Alkaline phosphatase
Cholesterol	Alanine aminotransferase
Glucose	Aspartate aminotransferase
Creatinine	Creatine kinase
Urea nitrogen	
Total bilirubin	

preparations were given in a volume of 10 ml/kg. The intubation tubes were flushed with 5 ml of water to ensure complete transfer. Individual doses were calculated based on daily recorded body weights. The test material mixtures were maintained during dose administration using a magnetic stir plate and stir bar.

Observation of animals. Each animal was observed twice daily (a.m. and p.m.) for signs of poor health or abnormal behavior. They were similarly observed at approximately 1 hour after each dose had been given. Effects were recorded as they were observed. Individual body weights were recorded weekly before treatment, on each dosing day, and on the day of sacrifice. Food consumption was confirmed qualitatively via daily inspection beginning at least one week before initiating treatment.

Clinical and anatomical pathology. After fasting overnight (water was provided ad libitum), each animal was bled from the femoral vein on day -7 (before initiating treatment), pre-dose on day 7, and on day 12 (at sacrifice). Table 1 gives a list of blood constituents measured.

On day 12, after overnight fasting, the animals were weighed, anesthetized with pentobarbital, exsanguinated, and necropsied in random order. The necropsy included a macroscopic examination of the external body surface, all orifices, cranial cavity, brain, spinal cord, nasal cavity and paranasal sinuses, viscera, and the thoracic, abdominal, and pelvic cavities. Tissues were preserved in phosphate-buffered formalin, imbedded in paraffin, sectioned and stained with hematoxylin and eosin, and examined microscopically.

RESULTS

Survival and antemortem observations. All four animals survived to the sceduled sacrifice on day 12. No adverse changes related to the test material were observed when 7-ODA was administered at 250 or 500 mg/kg; however, at 1,000 mg/kg excessive salivation was observed on day 9 in all animals before dosing, on day 10 in one male and both females immediately after dosing, and on day 11 in one male and both females before dosing. Both females vomited on days 2 and 4, and at 1,000 mg/kg each of the animals vomited at varying times. There were no testmaterial-related effects on body weight. Food consumption for all animals was normal with the exception of decreased consumption by both females on day 4 and for three out of four animals during the repeated dose phase at 1,000 mg/kg daily.

Anatomical pathology. No abnormalities were noted except that both females had a diffuse alopecia on the hind limbs. One female was in estrus. Forty two different organs and tissues were examined; all appeared to be normal.

Clinical pathology. At day -7 the concentration and/or appearance of blood constituents were within the normal range for young adult rhesus monkeys. After treatment was initiated there were several relatively small differences between pretreatment values and those on day 12 (post treatment). These differences were generally consistent for all animals studied and included lower red cell count, hemoglobin, and hematocrit and an increase in reticulocytes and platelets. The final concentrations were still in the normal ranges. No other hematological changes occurred.

Blood glucose increased from an average value of 70 mg/DL on day -7 to 100 on day 12; cholesterol decreased from 165 mg/DL to 124 and alkaline phosphatase decreased from 430 IU/L to 189 during the same time period. One female showed a striking increase in blood plasma creatine kinase. There were no significant alterations in the other blood constituents (Table I).

DISCUSSION

This study demonstrated that 7-oxo-DHEA-acetate (7-ODA) at dose levels of 250 and 500 mg/kg had no apparent adverse effects on rhesus monkey appearance, behavior, or body weight. The occurrence of lowered food intake at dose levels of 500 and 1,000 mg/kg did not result in body weight changes. Inconsistent effects of DHEA on body weight in animals and humans have been reported (9-15). In unpublished experiments (J. Kemnitz, H. Lardy et al.) feeding 140 mg of 7ODA/kg body weight daily to adult Rhesus monkeys for four weeks did not influence body weight or liver histology. Even that dose, smaller than used in the present study, is far greater than any amount likely to be used in humans.

Diffuse alopecia, observed at necropsy in both female monkeys, has also been seen in human females given DHEA (3).

The several relatively small differences in certain pre- and posttreatment blood values are of uncertain relationship to treatment. Confirmation of a definitive relation-

ship of these findings to 7- ODA was precluded by the absence of a control group which was omitted because of fund limitations. It is possible that some of the differences reflected normal day-to-day variability. None of these changes represented obvious health abnormalities or organ dysfunction, and values for individual animals were not atypical for young adult rhesus monkeys.

The occurrence of vomiting by both females on days 2 and 4 (when no active compound was administered) may indicate that vomiting at the higher dose was not caused by the steroid but by some other factor. The combination of steroid with the Tween detergent may have caused the reaction. 7-ODA taken for four weeks in doses of 100 mg bid by volunteers in a clinical trial did not cause nausia.

The increased liver mass found in rodents fed DHEA (16-19) was not manifest in our monkeys.

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7-oxo-DHEA and Raynaud's phenomenon

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Summary Patients with Raynaud's phenomenon have abnormal digital vasoconstriction in response to cold. The pathogenesis remains unknown but may involve a local neurovascular defect leading to vasoconstriction. Diagnosis of primary Raynaud's phenomenon is based on typical symptomatology coupled with normal physical examination, normal laboratory studies and lack of observable pathology by nail fold capillaroscopy. Secondary Raynaud's phenomenon is known to occur associated with several connective tissue diseases, vascular injury due to repeated vibrational trauma, and other causes which produce demonstrable vascular and microcirculatory damage. Treatment of Raynaud's symptoms is conservative and aimed at prevention of attacks. Patients are advised to remain warm and, if possible, to live in warm climates. We suggest that an ergogenic (thermogenic) steroid, 7-oxo-DHEA (3-acetoxyandrost-5-ene-7,17-dione), which is available without prescription as the trademarked 7-keto DHEA, may be very helpful in prevention of primary Raynaud's attacks by increasing the basal metabolic rate and inhibiting vasospasm.

INTRODUCTION

Raynaud's phenomenon is a common clinical disorder characterized by a recurrent, long-lasting, and episodic vasospasm of one or more digits, usually on exposure to cold. When the attack terminates, pallor and cyanosis of the affected digits is replaced by rubor due to reactive hyperemia. Maurice Raynaud in 1888 first described the disease as a local asphyxia and symmetrical gangrene of the extremity. Raynaud's phenomenon is classified as primary (formerly called Raynaud's disease) if a patient is noted to have vasospastic episodes precipitated by cold or emotional stress, but without evidence of any underlying medical illness during a 2-year observation period. Raynaud's phenomenon is classified as secondary (formerly Raynaud's syndrome or Raynaud's phenomenon) if there is an associated disease or disorder detected upon assessment. The distinction is important, because severity, treatment, and prognosis can all be affected.

Raynaud's phenomenon has a worldwide distribution, more noticeable in cold climates (1,2), with no racial predisposition, and a prevalence of 5–10% in the general population [(3), for a monograph, see (4)]. Primary Raynaud's disease usually occurs in young women aged between 11 and 45 years. The female to male ratio is about 4:1. The risk factors for primary Raynaud's phenomenon have not been fully determined, but well-known risk factors include living in cold climates, stress, female sex and a family history of primary Raynaud's phenomenon (5–7). Treatment is nonspecific since the underlying cause and the mechanism of vasospasm are unknown.

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CLINICAL ASPECTS AND DIAGNOSIS

Vasospastic attacks involve primarily the fingers, but may involve both fingers and toes. Involvement of only

the toes is much less frequent. Sometimes only one or few fingers may be affected during the attacks. Attacks can also involve nose, ears, nipples and lips, but rarely (8). Pallor, cyanosis, and subsequent rubor are the classic triphasic color changes that result from sudden vasoconstriction and subsequent reactive hyperemia. Many patients describe only pallor and rubor. Numbness of the digits caused by the lack of blood flow to the surrounding tissue usually accompanies pallor and cyanosis. Parathesia sometimes may be associated with the rubor. The duration of the attack may last from seconds to hours. If attacks are frequent and prolonged, thickening of the skin and brittle nails may occur and, in severe cases, ulceration may be observed. The most common precipitating factor is cold (9), either local cold or, significantly, whole body cooling. Emotions and stress can provoke attacks as well (10,11).

Since the diagnosis of primary Raynaud's disease is based entirely on typical symptomatology, the physician must elicit a careful historical description of the Raynaud's attacks, and as well a detailed personal and familial history must be taken, especially including drug history and smoke exposure. Before a diagnosis of primary Raynaud's disease is made, examination and interview should focus on determining whether other diseases, especially rheumatological and systemic disorders, are present, including sclerodermia, rheumatoid arthritis, and systemic lupus erythematosis. A history of hypertension or arthralgia should be taken. Signs of arthritis, skin ulcerations, telangiectasias, dyspnea, dysphagia, reflux, calcinosis, alopecia, oral ulcers, rash, serositis, and renal disease must be looked for. Cardiovascular disorders, angina, palpitations. intermittent claudication, atheroembolic and arterial obstructive disease should be investigated. An echocardiogram and arteriogram must be done to rule out atrial myxoma or arterial thrombosis. Hypercoagulable conditions such as the antiphospholipid antibody syndrome, history of miscarriage or cerebrovascular accidents with arterial or venous thrombosis should be investigated. Importantly, the presence of pulses should be determined and thoracic outlet and carpal tunnel maneuvers should be also performed.

Currently the best criteria for the diagnosis of primary Raynaud disease are the following: (1) absence of any disease or abnormality that could be responsible for vasospastic attacks; (2) well-demarcated digital pallor or cyanosis occurring in intermittent attacks, induced by cold or emotion; (3) symmetrical or bilateral involvement of the extremities; (4) gangrene, if present, usually limited to small areas of skin; (5) absence of antinuclear antibodies, normal erythrocyte sedimentation rate, normal nail fold capillaroscopy and esophageal motility studies (3).

THERAPY

The initial and most conservative therapeutic approach is focused on avoidance of precipitating factors, combined with ensuring warmth. Patient education and lifestyle changes are necessary, including cessation of smoking and avoidance of vasoconstriction drugs, such as caffeine, amphetamines, and pseudoephedrine. Alternative therapies, relaxation training, biofeedback, and acupuncture (12), may also be used to reduce stress, for patients whose attacks are correlated with stress.

If the attacks are severe and these preventive measures are insufficient or impractical, drug treatment is often necessary. A major problem with drug therapy is drug-induced side effects, exacerbated by the necessity of taking the drug continuously, at least throughout the cold months, in order to prevent infrequent attacks. Several classes of drugs with vasodilator action are available, including calcium channel blocking agents, antiadrenergic drugs, direct-acting vasodilators, beta agonists, prostaglandins, thromboxane synthetase inhibitors, and serotonin antagonists. Calcium channel blockers, which inhibit contraction of vascular smooth muscle by preventing calcium transport through the slow channel, are usually considered first line drug therapy (13). Side effects include tachycardia, headache, flushing and edema. Nifedipine, a dihydropyridine derivative is most commonly used (14) and preferred because of its selectivity on vascular smooth muscle and reduced effects on cardiac function. Antagonism of the alpha-adrenergic receptors via selective and nonselective blockade has been tried with variable success, often limited by such side effects as syncope, palpitations, headache, and weakness. Nonspecific vasodilators such as nitrates, which have a more pronounced effect on venules than arterioles, have some benefit in reducing the severity and frequency of attacks (15). Transcutaneous administration of nitric oxide has been effective in short-term trials (16). Immediate results with sympathectomy, a surgical option, are sometimes excellent, but vasospastic attacks usually recur within 6 months to 2 years (17,18). Complications such as Horner's syndrome, dry skin, pleural effusions, pneumothorax and atrial fibrillation may occur. Despite the availability of an ever-increasing number of therapeutic agents, most of the patients with Raynaud's disease continue to suffer from the disease.

T3 IS BENEFICIAL IN RAYNAUD'S

Reports from Peacock (19) and Dessein and Gledhill (20,21) indicated that the use of triiodothyronine (T3) in Raynaud's phenomenon could produce beneficial results. There are also several other case histories relevant

to thyroid hormone in the literature. A 50-year old with a 15 year history of Raynaud's phenomenon developed thyroid deficiency with exacerbation of the symptoms of Raynaud's. After substitution therapy with L-thyroxine, the patient became euthryroid and the symptoms of Raynaud's phenomenon disappeared (22). Similarly, several patients with Raynaud's phenomenon were found to be hypothyroid and replacement therapy again eliminated the symptoms (23,24). Kontos (25) in Cecil Textbook of Medicine states about T3 in Raynaud's, 'The resultant hypermetabolism elicits thermoregulatory reflex cutaneous vasodilatation'.

A successful controlled trial comparing 80 μg T3 with placebo was reported by Dessein et al (26). After 3 weeks, the frequency of attacks was reduced 4-fold, the duration reduced from 104 to 79 min in patients who still had attacks after T3 therapy, and attacks characterized as severe were reduced from 42 to 10%. Four of the six subjects had healing of digital ulcers. After therapy, about a 1 °C increase in skin temperature was noted over the skin creases of the digits and the palmar crease and the time to skin temperature recovery after immersion in ice water was shortened. No data are available on long term follow-up.

It is important to note that the patients in this T3 trial were euthryoid. Raynaud's phenomenon can be seen in hypothyroidism, along with cold intolerance and a decreased basal metabolic rate, and indeed T3 is effective in treating the Raynaud's component of hypothyroidism. In one report, 15 of 17 patients with myxoedema had cold hands (a common observation in myxoedema) and 4 had Raynaud's phenomenon; after therapy, those with cold hands decreased from 15 to 2 and only 1 of the 4 Raynaud's patients continued to have attacks. The Raynaud's patients all had closure of the digital arteries in a finger cooled to 0 °C before therapy, but only one after L-thyroxine treatment for 9 months (27). However the vast majority of patients with secondary Raynaud's phenomenon have some other condition than hypothyroidism (28).

It is also important to note that 16 of 18 patients in these T3 trials had associated disease, and only two were primary Raynaud's phenomenon. Other modestly successful clinical trials of other therapeutic agents suggest that primary and secondary Raynaud's phenomenon may not always respond equally well to therapy. A trial of nicardipine improved symptoms primary Raynaud's phenomenon in 12 patients, but not in 15 patients with systemic lupus erythematosus, progressive systemic sclerosis or rheumatoid arthritis (29). Similarly, a trial of diltiazem improved primary Raynaud's phenomenon, but less so for patients with systemic disease (30). The existence of demonstrable vascular damage might limit the effectiveness of an approach based solely on thermogenesis, although that was not the case in the T3 trial.

Because of the complex actions of T3, these experiments leave uncertain the explanation for the beneficial results obtained, although increased thermogenesis in certainly plausible given the role of thyroid hormone in basal metabolism and thermogenesis. An experiment in humans showed that mild hyperthyroidism induced with T3 increased whole body oxygen consumption by 17%; muscle TCA cycle flux was increased by about 70% without altering the rate of ATP synthesis (31).

ERGOGENIC STEROIDS

Ergogenic steroids may hold promise for the treatment of Raynaud's disease. These steroids act hormonally to increase heat production by mitochondria during ATP synthesis in humans and animals. The body temperature however does not rise significantly, because other thermoregulatory mechanisms maintain the temperature at a constant value and if necessary dispose of the excess heat by increasing blood flow to surfaces and to the periphery. It is the increased blood flow to the periphery that we believe will be helpful in Raynaud's disease.

During cold exposure, the normal acute physiological response is vasoconstriction, to reduce heat flow to the skin and heat loss. In response to cold, peripheral vasoconstriction begins below 35 °C and is maximal at 31 °C or less (32); decreased perfusion results in faster cooling and skin temperature decreases rapidly. Eventually, in the hands and fingers, cold-induced vasodilatation. under local and neural control is superimposed (33), but patients with Raynaud's phenomenon developed cold-induced vasoconstriction even after digital sympathectomy. There is a high variability between individuals for cold-induced vasodilatation. After cold-induced vasodilatation occurs, the skin temperature fluctuates, indicating a slow feedback process, prone to overshoot. Emotional stress can counteract the vasodilatation and lead to vasoconstriction [reviewed in (33)].

The palmar arch, which directly connects the ulnar and radial arteries, supplies the two digital arteries to the third, fourth and fifth fingers. The thumb and radial side of the index finger is supplied by the radial artery. The fingers contain many arteriovenous anastomoses which are involved in vasomotor activities (34), which are related to cold-induced vasodilatation [and which function abnormally in schleroderma (35)]. Flow through the capillary bed is a function both of local (capillary) factors and arteriovenous vasoregulation. It seems clear that a Raynaud's attack caused by failure of one or more of the regulatory mechanisms which control blood flow in response to skin temperature can be evaded by simply keeping the hands warm.

METABOLIC THERMOGENESIS

In humans, adaptive mechanisms, other than increased muscular activity, to acutely increase metabolic thermogenesis, are thought to be either weak or non-existent. However, muscular activity is very effective-shivering can increase the metabolic rate more than 4-fold and serious exercise 10–15-fold (36). Historical anecdotes of people surviving extreme conditions through muscular activity, such as walking are well known. Increasing the muscular activity of the hands and fingers during a Raynaud's attack is usually helpful.

Muscle and possibly liver, more so than adipose tissue, may the important tissues in humans for metabolic thermogenesis. For example, T3 increased muscle TCA cycle flux by about 70% without altering the rate of ATP synthesis (31). In that experiment, whole body oxygen consumption increased by 17%, and about 40% could be accounted for by muscle. In rodents, adaptive (non-shivering) metabolic thermogenesis is provided by innervated brown adipose tissue (BAT). These specialized adipocytes provide adaptive thermogenesis through accelerated lipolysis and fatty acid oxidation in mitochondria uncoupled from ATP synthesis. In BAT, there is a complex synergistic interaction between T4/T3 and norepinephrine (37). It is believed that in humans, BAT may be important in newborn thermogenesis, but not after infancy. Supporting this conclusion, the action of beta 3-adrenergic receptor agonists on BAT (thermogenesis) and white adipose tissue (lipolysis) are far more effective in weight reduction in rodents than in humans.

Although humans lack the adaptive BAT mechanism, this does not mean that increased thermogenesis is not possible in humans. Increased thermogenesis would presumably be manifested as an increase in basal metabolic rate.

ERGOGENIC PROPERTIES OF 7-OXO-DHEA

High dose DHEA induces thermogenesis, decreases body fat without decreasing food intake, and decreases glucose levels in ob/ob or db/db diabetic mice (38). Resting heat production in rats is increased by DHEA (39) Derivatives of DHEA were investigated for their ergogenic properties, and 7-oxo-DHEA in particular was shown to be 4-fold more thermogenic than DHEA. In vivo, DHEA is converted to 7-α-hydroxy-DHEA and then to 7-oxo-DHEA, with further metabolism to 7-β-hydroxy-DHEA(40). Both the hydroxy and keto derivatives of DHEA are more thermogenic than DHEA itself.

The thermogenic properties of DHEA and 7-oxo-DHEA are likely to be due to two interrelated actions, increased levels of enzymes and proteins that shuttle substrate and electrons in and out of the mitochondria, and an increased proton leak across the mitochondrial inner membrane. Experimental evidence for increased shuttling by DHEA and 7-oxo-DHEA is much better than for induction of a proton leak. Levels of malic enzyme, which converts pyruvate to malate, can be increased more than 5-fold by 7-oxo-DHEA (41) and more than 4-fold by DHEA (42), Malic enzyme is also under T3 control, but the increase of liver malic enzyme by DHEA was shown to be additive to that of thyroid hormone (43) Malate can enter the mitochondrion to expand the supply of TCA intermediates and can transfer reducing equivalents as part of the malate-asparate shuttle. Levels of cytoplasmic glycerol 3-P dehydrogenase can be increased 3.3–3.7-fold (40,41) and result in increased activity of the glycerol 3-P shuttle which transfers electrons from cytoplasm to form FADH2 in the mitochondrial matrix. Four times as much DHEA is required to produce the same level of changes that are induced by 7-oxo-DHEA (41). The activity of ATP:ADP translocase is also increased.

By themselves, increased shuttle enzymes might not be very thermogenic, since under most circumstances, including exposure to cold, the rate of mitochondrial oxidation is determined by the need to synthesize ATP, that is by the levels of ADP. However, mitochondria from rats treated with 7-oxo-DHEA appear to have an increased proton leak, suggesting that it may elevate levels of uncoupling proteins (44), and thereby lead to increased uncoupling between ATP synthesis and mitochondrial oxidation.

Uncoupling proteins

Uncoupling proteins provide a pathway for the entry of protons into the mitochondrial matrix without driving ATP synthesis. Intramitochondrial synthesis of ATP is catalyzed by ATP synthases coupled to and energetically driven by the influx of protons across the inner mitochondrial membrane. Influx of protons through ATP synthases occurs in response to an electrochemical gradient established by the export of protons by proton pumps coupled to the oxidation of the coenzymes FADH2 and NADH. To the extent that the electrical gradient is dissipated by entry of protons not coupled to ATP synthase, free energy from the oxidation of substrate is converted entirely to heat, instead of being partially conserved as ATP to be used in subsequent biochemical reactions. As a result, more substrate is oxidized (and more heat is produced) in the process of producing the same amount of ATP.

Three uncoupling proteins are known. UCP-1 is expressed exclusively in brown adipose tissue, in which levels of UCP-1 are considerably increased by cold exposure and where UCP-1 clearly has the role of

increasing heat production by uncoupling respiration from ATP synthesis. In rodents, thermogenesis in brown adipocytes, which are innervated, can be activated by release of norepinephrine from nerve fibers. Loss of this thermogenic system by targeted disruption of the gene for type 2 iodothyronine deiodinase (37) or the gene for UCP-1 (45) forces the mice to survive by shivering. In humans the amount of BAT is small and is greatly reduced after infancy; its role in human adaptive thermogenesis is unfortunately not clear at present. UCP2 is expressed widely in human tissue and UCP3 is expressed selectively in human skeletal muscle, but their roles in thermogenesis are also not clear. Generally however, these three proteins are believed to be intimately intertwined with energy and weight regulation, substrate selection, insulin release and other related physiological parameters.

Synthesis of all uncoupling proteins is increased by exposure to cold and is increased by the hormones leptin and T3 (46), and also adrenergic drugs, PPAR stimulators, and TNF-α. Insulin increases the levels of UCP-1 only. Levels of all three uncoupling proteins are decreased by glucocorticoids which are well known to influence glucose levels and lipid deposition. Glucocorticoids also strongly inhibit the ability of adrenergic stimulation to increase levels of UCP-1 (47). Many of the properties of DHEA and its derivatives such as 7-beta-hydroxy-DHEA (48) are anti-glucocorticoid in nature, including the effect on UCP.

Mitochondria from rats treated with 7-oxo-DHEA appear to have an increased proton leak, suggesting that it may elevate levels of uncoupling proteins (44). It seems likely that 7-oxo-DHEA induces synthesis of higher levels of uncoupling proteins, especially UCP-1, but perhaps also UCP-2 and/or UCP-3. In humans, the importance of these uncoupling proteins to thermogenesis is not certain However, 7-oxo-DHEA is certainly thermogenic in humans. It seems unlikely, but possible, that this thermogenesis is due only to the increased levels of shuttle enzymes, although increasing the level of proteins needed to shuttle substrates and reducing equivalents into the mitochondria can certainly assist in thermogenesis.

Although we are here primarily concerned with thermogenesis, it should be noted that these drugs alter the relative activity of pathways as well. Isolated hepatocytes from rats treated with high levels of DHEA produced 2.5-fold more CO_2 from glucose than control rats. Gluconeogenesis from alanine was reduced to one-half (42). In this experiment, the reduction in gluconeogensis and the increase in glycolysis may be a consequence of the utilization of the gluconeogenic precursors by the hyperactive mitochondria, although it could also reflect a rise in AMP levels, or some other cause. The antidiabetic drug metformin is well known also to inhibit gluconeogenesis and to have modest weight-reducing properties. Metformin may act by inhibition of complex I (49,50). Complex I utilizes NADH, equivalent to 3 ATP, and Complex II utilizes FADH2, equivalent to 2 ATP, so inhibition of Complex I would be expected to decrease the efficiency of ATP synthesis.

SAFETY OF 7-OXO-DHEA

In humans, a safety and pharmacokinetic study in human males at 50, 100, and 200 mg/day showed that 7-oxo-DHEA was converted to 7-oxo-DHEA-S which had a half life of 2.17 h and did not accumulate (51). [In rats, the sulfated derivative is as active as 7-oxo-DHEA (52).] Levels of various relevant hormones that were monitored did not change and there were no adverse effects reported. In monkeys, doses of 250, 500, and 1000 mg/kg were given short term; on autopsy, no lesions or abnormalities were noted (53). In a related experiment, in rats a single oral dose of 2000 mg/kg demonstrated no adverse effects (54).

CONCLUSIONS

We suggest that increased peripheral vasodilation accompaning increased thermogenesis induced with 7-oxo-DHEA might provide the molecular equivalent of keeping the hands warm in Raynaud's disease. An initial result is encouraging. A 46-year old patient had increasingly frequent vasospastic attacks accompanied by pain and pallor in her left fingers except the thumb. Precipitating factors included being in an excessively air-conditioned environment, touching cold objects, and digging in cold, wet earth. The episodes were less than 10 min duration. The patient had no other complaints, denied other illnesses, and does not take any prescription medications. The patient does not smoke, and consumes minimal amounts of alcohol occasionally. On examination, her hands were without any abnormalities with normal skin and color appearance. These vasospastic attacks did not occur after taking 7-oxo-DHEA but returned within one week of discontinuing the medication, and disappeared again on resuming the medication.

It would appear to us worthwhile to conduct a randomized, placebo-controlled, cross-over clinical study of the response to 7-oxo-DHEA in patients with primary Raynaud's disease. These patients should be healthy, non-smoking normotensive, normocholesterolemic, normoglycemic controls with no evidence or history of autoimmune or vascular disease. A similar study of Raynaud's phenomenon secondary to autoimmune disease would be worthwhile, although the complexity of

controlling for the different precipitating factors and disease progression would make this a more challenging study.

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Oxidation of Cholesterol, 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one by Rat Liver Microsomes

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The metabolism of cholesterol, 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one and the formation of lipid peroxides from endogenous lipids were studied in rat liver microsomes fortified with NADPH. Under conditions of NADPH-dependent lipid peroxidation cholesterol was oxidized into several metabolites including 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol and cholestane-3β,5α,6β-triol. When lipid peroxidation was inhibited, cholesterol was converted predominantly into 5-cholestene-3β,7α-diol. Incubation of cholesterol with soybean lipoxidase and linoleic acid yielded the same pattern of products as incubation of cholesterol with microsomal fraction under conditions of NADPH-dependent lipid peroxidation. 3β-Hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one were metabolized by the microsomal fraction in essentially the same way whether or not lipid peroxidation was inhibited. The main metabolite of 3β-hydroxy-5-pregnen-20-one could not be identified. Gas-chromatographic and mass-spectrometric analyses indicated that it was a monohydroxy derivative of 3β-hydroxy-5-pregnen-20-one. Other hydroxylated metabolites of 3β-hydroxy-5-pregnen-20-one were 3β,7α-dihydroxy-5-pregnen-20-one, 3β,7β-dihydroxy-5-pregnen-20-one and 3β,16α-dihydroxy-5-pregnen-20-one. Major hydroxylated metabolites of 3β-hydroxy-5-androsten-17-one were 3β,7α-dihydroxy-5-androsten-17-one, 3β,7β-dihydroxy-5-androsten-17-one and 3β,16α-dihydroxy-5-androsten-17-one.

Biliary drainage resulted in an eight-fold stimulation of the 7α-hydroxylation of cholesterol but inhibited all hydroxylations of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one. Phenobarbital treatment had no significant effect on the 7α-hydroxylation of cholesterol or the formation of lipid peroxides but significantly stimulated the formation of 3β,16α-dihydroxy-5-pregnen-20-one, 3β,7α-dihydroxy-5-androsten-17-one and 3β,7β-dihydroxy-5-androsten-17-one. All hydroxylations were inhibited by carbon monoxide. No significant inhibition of lipid peroxidation by carbon monoxide was observed.

The introduction of a 7α-hydroxyl group is the first and rate-limiting step in the biosynthesis of cholic acid from cholesterol [1]. This reaction as well as a large number of other hydroxylations are catalyzed by the microsomal fraction of liver fortified with NADPH and in the presence of oxygen [2—5]. An electron-transport chain including cytochrome P-450 as the terminal oxidase appears to be involved in many of these hydroxylations including the 7α-hydroxylation of cholesterol [4]. In a previous communication from this laboratory the enzymatic formation of 3β-hydroxy-5-cholesten-7-one from cholesterol in the presence of the microsomal fraction of rat liver homogenate fortified with NADPH was reported [6]. It was also found that the extent of conversion of cholesterol into 3β-hydroxy-5-cholesten-7-one was much lower in the presence of the 20000 × *g* supernatant fluid and that there appeared to be an inverse correlation between the extent of conversion of cholesterol into 3β-hydroxy-5-cholesten-7-one and into 5-cholestene-3β,7α-diol. The formation of 5-cholestene-3β,7α-diol was more efficient in the presence of the 20000 × *g* supernatant fluid than in the presence of the microsomal fraction [6]. The microsomal fraction of liver homogenate fortified with NADPH has been shown to catalyze the formation of lipid peroxides from some polyunsaturated fatty acids such as linoleic acid and linolenic acid, and it has been suggested that the processes of peroxidation and hydroxylation are closely related and may be a result of the operation of the same electron-transport chain [7—10]. Since lipid peroxidation resembles the conversion of cholesterol into 3β-hydroxy-5-cholesten-7-one in being much more efficient in the presence of microsomal fraction fortified with NADPH than in the presence of microsomal fraction plus the supernatant fluid, it appeared possible that there is a connection between the formation of lipid peroxides from endogenous

Nomenclature. The systematic name of cholic acid is 3α,7α,12α-trihydroxy-5β-cholanoic acid.

fatty acids and the formation of 3β-hydroxy-5-cholesten-7-one [11]. Recently, Wills [9] reported that when the microsomal fraction was prepared in a sucrose medium containing EDTA, the formation of lipid peroxides was inhibited efficiently. To obtain information on the possible relation between the formation of lipid peroxides and the oxidation of cholesterol in rat liver microsomes, the metabolism of cholesterol has been studied in fortified microsomal fractions prepared in the presence and absence of EDTA. Since the microsomal fraction catalyzes oxidation in the 7-position of Δ^5-3β-hydroxysteroids of the C_{19} and C_{21} series, it appeared of interest to include such steroids in the study.

EXPERIMENTAL PROCEDURE

Materials

[4-^{14}C]Cholesterol (specific radioactivity, 145 μCi/mg), 3β-hydroxy-5-[4-^{14}C]pregnen-20-one (1.2 μCi/mg) and 3β-hydroxy-5-[4-^{14}C]androsten-17-one (0.5 μCi/mg) were obtained from the Radiochemical Centre (Amersham, England). In experiments with soybean lipoxidase [4-^{14}C]cholesterol with a specific radioactivity of 10 μCi/mg and 3β-hydroxy-5-[4-^{14}C]-pregnen-20-one with a specific radioactivity of 0.6 μCi/mg were used. Prior to use, [4-^{14}C]cholesterol was purified by chromatography on a column of neutral aluminium oxide, grade III (Woelm, Eschwege, Germany). The other labeled compounds were purified by chromatography on columns of hydroxyalkoxypropyl-Sephadex with methanol—water—1,2-dichloroethane (7:3:1, v/v/v) as solvent [12].

5-Cholestene-3β,7α-diol (m.p. 183—185 °C, reported [13] m.p. 185 °C), 5-cholestene-3β,7β-diol (m.p. 172—173 °C, reported [14] m.p. 178 °C), 3β-hydroxy-5-cholesten-7-one (m.p. 172 °C, reported [15] m.p. 170—172 °C), 5-cholestene-3β,7α,12α-triol (m.p. 194 to 195 °C, reported [16] m.p. 194—195 °C) and cholestane-3β,5α,6β-triol (m.p. 231—234 °C, reported [17] m.p. 237—239 °C) were synthesized according to methods described previously [16,18]. 3β,7α-Dihydroxy-5-pregnen-20-one (m.p. 169—170 °C, $[\alpha]_D^{23}$ −46° (*c*, 0.4 in chloroform); reported [19] m.p. 190 °C, $[\alpha]_D$ −28°), 3β,7β-dihydroxy-5-pregnen-20-one (m.p. 188—190 °C, $[\alpha]_D^{23}$ +45° (*c*, 0.4 in chloroform)), 3β,7α-dihydroxy-5-androsten-17-one (m.p. 175 to 176 °C, $[\alpha]_D^{23}$ −70° (*c*, 0.4 in chloroform); reported [20] m.p. 181.5—183.5 °C, $[\alpha]_D$ −70.7°) and 3β,7β-dihydroxy-5-androsten-17-one (m.p. 184—185 °C, $[\alpha]_D^{23}$ +59° (*c*, 0.4 in chloroform); reported [20] m.p. 215 to 216 °C, $[\alpha]_D$ +67.5°) were prepared according to procedures described by Stárka [21] and were purified by chromatography on columns of hydrophobic Hyflo Super-Cel with phase system F1 [22]. The crystallized compounds were analyzed by thin-layer chromatography and as trimethylsilyl ethers by combined gas chromatography—mass spectrometry with the LKB 9000 instrument equipped with a 1.5% SE-30 column. These analyses confirmed the purity and identity of the synthesized compounds. Although the samples had been carefully dried, it is possible that the differences between the melting points reported for 3β,7α-dihydroxy-5-pregnen-20-one and 3β,7β-dihydroxy-5-androsten-17-one and those found in the present investigation are due to the presence of solvent in the crystals. 3β-Hydroxy-5-pregnene-7,20-dione (m.p. 208—211 °C; reported [23] m.p. 209 to 210 °C) and 3β-hydroxy-5-androstene-7,17-dione (m.p. 238—240 °C; reported [20] m.p. 243—244.5 °C) were prepared from 3β-hydroxy-5-pregnen-20-one 3-acetate and 3β-hydroxy-5-androsten-17-one 3-acetate, respectively, by oxidation with *tert*-butyl chromate followed by hydrolysis with potassium carbonate [24,25]. 3β,16α-Dihydroxy-5-pregnen-20-one (m.p. 250—252 °C; reported [26] m.p. 252—255 °C) was a generous gift of Dr. J.-Å. Gustafsson. 3β,16α-Dihydroxy-5-androsten-17-one (m.p. 174—175 °C; reported [27] m.p. 177—181 °C) was obtained from Sigma Chemical Co. (St. Louis, Mo.) NADH, NADPH, *cis*-linoleic acid and soybean lipoxidase (83000 units/mg) were obtained from Sigma Chemical Co.

Methods

Animal Experiments. Male rats of the Sprague-Dawley strain weighing 150—200 g were used. In experiments with rats with a biliary fistula the animals were starved but had free access to drink; saline for the rats with a biliary fistula and tap water for the control rats. These experiments lasted for 60 h. Phenobarbital (100 mg/kg body weight) in 1 ml of saline was administered intraperitoneally daily for 5 days. The animals were killed 24 h after the last injection. In the phenobarbital experiments the animals had free access to drink and a commercial pellet diet.

Preparation of Rat Liver Microsomes. Liver homogenates (20%, w/v) were prepared in 0.25 M sucrose or in 0.25 M sucrose containing 0.001 M EDTA using a Potter-Elvehjem homogenizer equipped with a loosely fitting teflon pestle. The homogenate was centrifuged at 800×*g* for 10 min and at 20000×*g* for 10 min. The 20000×*g* supernatant fluid was centrifuged at 100000×*g* for 1 h. The resulting pellet was drained carefully and suspended in 0.1 M potassium phosphate, pH 7.0, containing 0.028 M nicotinamide. In some experiments 0.1 M Tris-Cl buffer, pH 7.0, was used. The microsomal fraction was suspended by homogenizing with a loosely fitting pestle in a volume corresponding to the original 20000×*g* supernatant. In experiments with variations of pH the microsomal fraction was suspended in 0.125 M potassium chloride. Boiled microsomal fraction was obtained by heating the microsomal suspension at 80 °C for 5 min followed by centrifugation at 800×*g* for 10 min.

Experiments with Rat Liver Microsomes

Incubations with [4-^{14}C]Cholesterol. Microsomal fraction, 3 ml; buffer, 2 ml; [4-^{14}C]cholesterol, 15 µg dissolved in 50 µl acetone; and NADPH, 5 µmoles; were incubated for 15 min at 37 °C. Incubation was terminated by the addition of 20 volumes chloroform—methanol (2:1, v/v). The precipitate was filtered off and 0.2 volumes of a 0.9% (w/v) sodium chloride solution were added. The residue of the chloroform phase together with appropriate reference compounds as internal standards was subjected to thin-layer chromatography with Kieselgel G (Merck, Darmstadt, Germany) as adsorbent and benzene—ethyl acetate (2:3, v/v), as solvent. The internal standards were visualized by iodine vapor. The iodine was evaporated at room temperature, the appropriate zones were scraped into test tubes and extracted with 5 ml methanol by vigorous stirring. The silica gel was allowed to settle by gravity and 1 ml of the methanol solution was evaporated in counting vials and assayed for radioactivity.

Incubations with 3β-Hydroxy-5-[4-^{14}C]pregnen-20-one. Microsomal fraction, 1 ml, and 3 µmoles NADPH were diluted with buffer to 3 ml. In experiments with phenobarbital-treated rats and the corresponding control rats, 0.5 ml of microsomal fraction was used. 3β-Hydroxy-5-[4-^{14}C]pregnen-20-one, 100 µg dissolved in 50 µl of acetone, was added and incubation was carried out for 10 min at 37 °C. The incubation was terminated, extracted and worked up as described above but with 2,2,4-trimethylpentane-isoamyl acetate—acetone (2:2:1, v/v/v), as solvent for thin-layer chromatography. The chromatoplates were run three times in the same solvent with drying of the plates between the runs.

Incubations with 3β-Hydroxy-5-[4-^{14}C]androsten-17-one. Microsomal fraction, 0.5 ml, and 3 µmoles of NADPH were diluted with buffer to 3 ml. 3β-Hydroxy-5-[4-^{14}C]androsten-17-one, 200 µg dissolved in 50 µl of acetone, was added. Incubation was carried out for 10 min at 37 °C. Analysis was performed as described above for incubations with 3β-hydroxy-5-[4-^{14}C]pregnen-20-one.

Assay of Lipid Peroxide Formation. To measure the rate of formation of lipid peroxides, samples of 0.5 ml were taken every fifth min from incubations with [4-^{14}C]cholesterol. The content of lipid peroxides in the samples was estimated by determining malonaldehyde with the thiobarbituric-acid method as described by Wilbur, Bernheim and Shapiro [28]. The molar absorption coefficient $\varepsilon_{530} = 1.56 \times 10^5\ M^{-1} \times cm^{-1}$ [29] was used.

Determination of Protein and Cholesterol. Protein was determined according to Lowry *et al.* [30]. The protein concentration of the microsomal fraction varied between 2 and 3.5 mg per ml. Cholesterol was determined as described by Hanel and Dam [31].

Experiments with Soybean Lipoxidase

[4-^{14}C]Cholesterol, 3β-hydroxy-5-[4-^{14}C]pregnen-20-one or 3β-hydroxy-5-[4-^{14}C]androsten-17-one, 200 µg dissolved in 50 µl of acetone, was suspended in 1.5 ml of 0.1 M Tris-Cl buffer, pH 7.5. Soybean lipoxidase, 100 µg (8300 units) dissolved in 0.1 ml of 0.1 M Tris-Cl buffer, pH 7.5, and/or 400 µg of linoleic acid were added and the mixture was shaken for 10 min at 30 °C. Prior to addition, the linoleic acid was converted into its ammonium salt by the addition of 0.25 ml 0.019 M NH_4OH/mg linoleic acid. Further analysis was carried out as described above for the different substrates.

Radioactivity Assay

Radioactivity was measured with a Packard scintillation spectrometer, model 4322, with a counting efficiency for ^{14}C of 73%. The scintillant consisted of a solution of 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene in 1 l of toluene.

Statistical Analysis

The Student *t*-test was used and the significance level was set at 0.01.

RESULTS

Oxidation of Cholesterol by Rat Liver Microsomes Prepared in Sucrose Medium

Fig. 1 A shows a thin-layer chromatogram of the chloroform extract of an incubation of [4-^{14}C]cholesterol with microsomal fraction prepared in sucrose medium and fortified with NADPH. The major products were identified as 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol and cholestane-3β,5α,6β-triol by crystallization to constant specific radioactivity together with the authentic compounds (for details concerning these results see Annex Table 1). As is evident from the thin-layer chromatogram shown in Fig. 1 A several other products were formed. No attempt was made to identify these products. The results of the crystallizations also showed the presence of small amounts of other unknown compounds. The formation of all products required the addition of NADPH to the microsomal fraction (Fig. 1 B) and was reduced by about 85% by boiling the microsomal fraction. Fig. 2 shows the effect of various factors on the conversion of cholesterol into 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol. The formation of lipid peroxides was measured simultaneously. Oxidation of cholesterol as well as formation of lipid peroxides were linear with time for at least 20 min (Fig. 2 A). The rate of oxidation of cholesterol was constant between 0.5 and 1.5 mg of microsomal protein per ml. It should be pointed out

	A	B	C
Front			
5	2 751 411	2 054 290	1 718 667
	13 643	1798	2287
4	74 079	1845	1708
3	17 905	1263	1043
2	15 678	961	8389
	2601	895	474
1	15 526	868	1339
Start	14 849	1915	2371

Fig. 1. *Thin-layer chromatograms of extracts of incubations of [4-^{14}C]cholesterol with microsomal fraction.* Three ml of microsomal fraction, prepared in sucrose (A and B) or in EDTA-sucrose (C) were diluted with buffer to 5 ml and incubated for 15 min with 15 μg of [4-^{14}C]cholesterol. In experiments shown in A and C, 5 μmoles of NADPH were added to the incubations. The numbers on the chromatograms represent counts/min. Reference compounds were: (1) cholestane-3β,5α,6β-triol; (2) 5-cholestene-3β,7α-diol; (3) 5-cholestene-3β,7β-diol; (4) 3β-hydroxy-5-cholesten-7-one; (5) cholesterol. Solvent, benzene—ethyl acetate (2:3, v/v)

that the amount of [4-^{14}C]cholesterol added to the incubations is small in comparison with the amount of cholesterol present in the microsomal fraction and that estimation of cholesterol oxidation is based on radioactivity. The rate of formation of lipid peroxides decreased with increasing protein concentration (Fig. 2B). Between 0.5 and 1.5 mg of microsomal protein per ml, the total amount of lipid peroxides formed was constant and was thus not influenced by protein concentration. The effect of varying concentrations of NADPH on oxidation of cholesterol and formation of lipid peroxides is shown in Fig. 2C. The rate of reaction with NADH was less than 10% of that with NADPH. NADPH could be replaced by ascorbate but the rate of reaction was much slower (Fig. 2D). The pH-optimum for oxidation of cholesterol as well as formation of lipid peroxides was about 6.5 with NADPH as cofactor (Fig. 2E) and about 6.0 with ascorbate (Fig. 2F). With NADPH as cofactor cholesterol oxidation and lipid-peroxide formation were stimulated about four-fold by addition of Fe^{2+} at a concentration of 0.01 mM and about six-fold by Fe^{2+} at a concentration of 0.1 mM.

Oxidation of Cholesterol by Rat Liver Microsomes Prepared in EDTA-Sucrose Medium

Fig. 1C shows a thin-layer chromatogram of the chloroform extract of an incubation of [4-^{14}C]cholesterol with microsomal fraction prepared in EDTA-sucrose medium and fortified with NADPH. The predominant product was identified as 5-cholestene-3β,7α-diol (Annex, Table 2). Only small amounts of 3β-hydroxy-5-cholesten-7-one and 5-cholestene-3β,7β-diol were formed (Fig. 1C). The amounts of lipid peroxides formed were very small and were estimated to be less than 2% of those formed in incubations of microsomal fraction prepared without EDTA. The rate of conversion of cholesterol into 5-cholestene-3β,7α-diol was linear with time for at least 15 min (Fig. 3A). The rate of reaction was constant between 0.5 and 1.5 mg of microsomal protein per ml (Fig. 3B). The effect of varying concentrations of NADPH is shown in Fig. 3C. The rate of reaction with NADH was less than 10% of that with NADPH. The pH-optimum for the 7α-hydroxylation of cholesterol was about 7.0 (Fig. 3D). The effect on the reaction of various divalent metal ions is summarized in Table 1. All metal ions were added as chloride salts except Fe^{2+} which was added as sulfate. Sulfate as such had no effect on the reaction. In these experiments the microsomal fraction was suspended in Tris-Cl buffer instead of phosphate buffer. The rate of 7α-hydroxylation in Tris-Cl buffer was about half of that in phosphate buffer. The 7α-hydroxylation was stimulated by Fe^{2+} ions and inhibited by Cu^{2+} and Hg^{2+} ions. The stimulation of the 7α-hydroxylation by Fe^{2+} ions was accompanied by stimulation of the formation of 3β-hydroxy-5-cholesten-7-one and 5-cholestene-3β,7β-diol and of lipid peroxides. In fact, the oxidation of cholesterol and the formation of lipid peroxides in the presence of 0.1 mM Fe^{2+} ion proceeded as with microsomal fraction prepared without EDTA in the homogenizing medium. The effects of Zn^{2+} and Ni^{2+} ions were similar to those of Fe^{2+} ions but were less pronounced.

Oxidation of Cholesterol by Lipid Peroxides

[4-^{14}C]Cholesterol was incubated with soybean lipoxidase in the presence of linoleic acid. Fig. 4A shows a thin-layer chromatogram obtained from such an incubation. Several labeled products were formed including 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol (Annex, Table 3). No attempt was made to identify the other products. Without linoleic acid and/or lipoxidase very small amounts of oxidized products were formed (Fig. 4B—D).

Oxidation of 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one by Rat Liver Microsomes

Fig. 5 shows thin-layer chromatograms of chloroform extracts of incubations of 3β-hydroxy-5-[4-^{14}C]-pregnen-20-one and 3β-hydroxy-5-[4-^{14}C]androsten-17-one with the microsomal fraction prepared in EDTA-sucrose medium, with and without NADPH

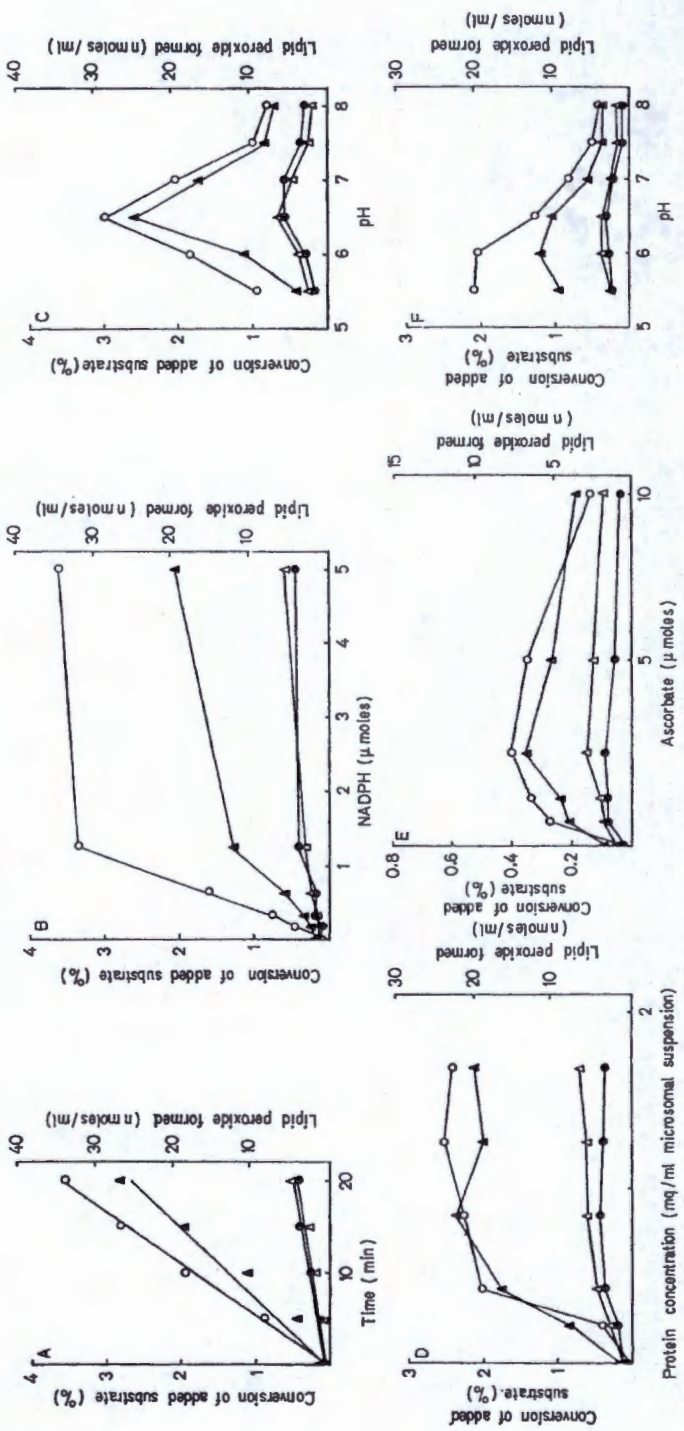

Fig. 2. Effect of time (A), amount of microsomal fraction (D), NADPH (B), ascorbate (E) and pH (C, F) on oxidation of cholesterol and formation of lipid peroxides by microsomal fraction prepared in sucrose. The standard incubation mixture consisted of 3 ml of microsomal fraction, 5 µmoles of NADPH and buffer, pH 7.0, to a final volume of 5 ml. This mixture was incubated for 15 min with 15 µg of [4-^{14}C]cholesterol. In D and F, NADPH was substituted with 2.5 µmoles of ascorbate. O, Formation of lipid peroxides, in nmoles of malonaldehyde formed per ml microsomal suspension; ▲, formation of 3β-hydroxy-5-cholesten-7-one; ●, formation of 5-cholestene-3β,7α-diol; △, formation of 5-cholestene-3β,7β-diol

added to the incubations. The main products isolated had the chromatographic properties of the corresponding 7-oxo, 7α-, 7β- and 16α-hydroxy derivatives. At least one other labeled compound was formed from both 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one. No attempt was made to identify these compounds. The identity of the 7-oxo, 7α-, 7β- and 16α-hydroxy compounds was established by crystallization to constant specific radioactivity together with the authentic compounds (Annex,

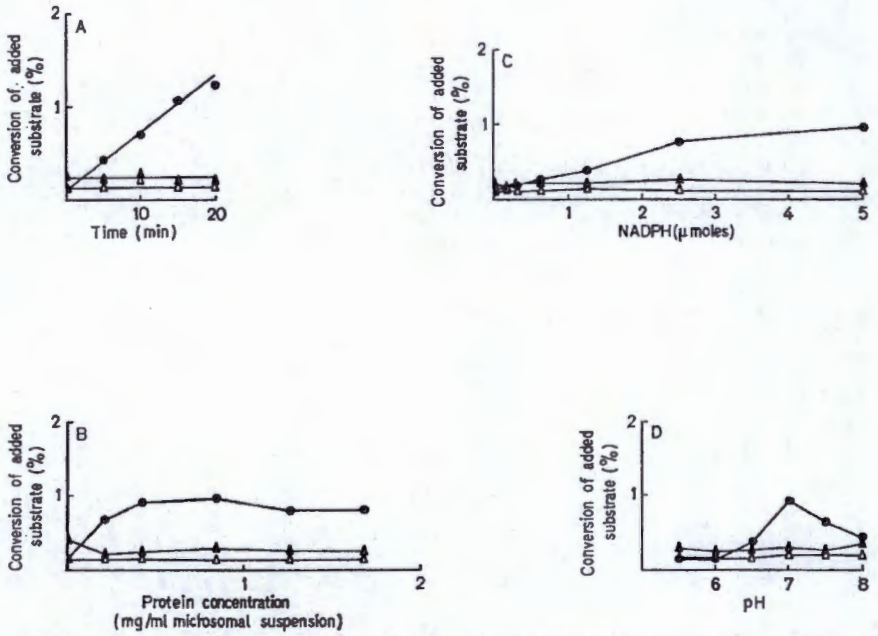

Fig. 3. *Effect of time (A), amount of microsomal fraction (B), NADPH (C) and pH (D) on oxidation of cholesterol by microsomal fraction prepared in EDTA-sucrose.* The incubation mixture and the incubation conditions were as those given in legend to Fig. 2. ▲, Formation of 3β-hydroxy-5-cholesten-7-one; ●, formation of 5-cholestene-3β,7α-diol; △, formation of 5-cholestene-3β,7β-diol

Table 1. *Effect of metal ions on oxidation of cholesterol and formation of lipid peroxides by microsomal fraction prepared in EDTA-sucrose*

Metal ion was added as chloride salt except Fe^{2+} which was added as sulfate. The concentration of metal ion in the incubation mixtures was in all instances 0.1 mM. The percentages were calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms. The formation of lipid peroxides is expressed in the amount of malonaldehyde formed per ml of microsomal suspension (*cf.* Fig. 2B)

Metal ion added	Conversion of cholesterol into			Formation of lipid peroxides
	5-Cholestene-3β,7α-diol	5-Cholestene-3β,7β-diol	3β-Hydroxy-5-cholesten-7-one	
	% of added substrate			nmoles/ml
None	0.23	0.08	0.16	< 0.5
Mg^{2+}	0.22	0.09	0.14	< 0.5
Ca^{2+}	0.26	0.08	0.16	< 0.5
Mn^{2+}	0.18	0.08	0.10	< 0.5
Fe^{2+}	0.64	0.64	2.76	27.4
Co^{2+}	0.21	0.08	0.16	< 0.5
Ni^{2+}	0.23	0.12	0.31	0.7
Cu^{2+}	0.13	0.07	0.12	< 0.5
Zn^{2+}	0.22	0.20	0.86	7.7
Hg^{2+}	0.12	0.09	0.14	< 0.5

Tables 4 and 5). The crystallization data showed that only about 15% of the labeled material in the zone corresponding to 3β,7β-dihydroxy-5-pregnen-20-one was identical with this compound. Labeled material with the thin-layer chromatographic properties of 3β,7β-dihydroxy-5-pregnen-20-one was converted into the trimethylsilyl ether and analyzed by radio-gas chromatography and combined gas chromatography—mass spectrometry. Radio-gas chromatography (Fig. 6) showed that most of the radioactivity (peak II) appeared after 3β,7β-dihydroxy-5-pregnen-20-one (peak I). The mass spectrum of the unknown labeled compound indicated that the compound was a dihydroxy-5-pregnen-20-one. The base peak was at *m/e* 386 (M-90, loss of trimethylsilanol). Peaks were seen at *m/e* 461 (M-15), *m/e* 371 (M-(90 + 15)), *m/e* 343 (M-(90 + 43), loss of trimethylsilanol and side chain) and *m/e* 296 (M-2×90, loss of two molecules of trimethylsilanol). There was a small peak at *m/e* 476 (M). It appears reasonable to

	A	B	C	D
Front				
5	2 235 420	2 659 570	2 584 720	2 245 505
	21 618	4088	2892	2683
4	29 131	3512	3706	3090
3	11 160	1413	1421	1241
2	10 054	1655	1518	858
	3 259	764	725	225
1	8254	1681	1858	552
Start	4737	2691	4131	1451

Fig. 4. *Thin-layer chromatograms of extracts of incubations of [4-^{14}C]cholesterol with soybean lipoxidase and linoleic acid.* (A) [4-^{14}C]cholesterol, 200 µg, 100 µg of soybean lipoxidase and 400 µg of linoleic acid were added to 1.5 ml of 0.1 M Tris-Cl buffer, pH 7.5, and the mixture was incubated at 30 °C for 10 min; (B) same as in A with the omission of lipoxidase; (C) same as in A with the omission of linoleic acid; (D) same as in A with the omission of soybean lipoxidase and linoleic acid. The numbers on the chromatograms represent counts/min. Reference compounds were: (1) cholestane-3β,5α,6β-triol; (2) 5-cholestene-3β,7α-diol; (3) 5-cholestene-3β,7β-diol; (4) 3β-hydroxy-5-cholesten-7-one; (5) cholesterol. Solvent, benzene—ethyl acetate (2:3, v/v)

	A	B	C	D
Front				
5	150 038	127 697		
	1460	3626		
4	302	4753		
3	0	4435		
2	106	13 272		
1	195	2342		
	187	1328		
Start	354	2544		

	C	D
Front		
10	110 926	109 472
	851	3731
9	406	2696
	178	472
8	384	1247
	129	664
7	232	1533
6	166	9584
	97	285
Start	338	394

Fig. 5. *Thin-layer chromatograms of extracts of incubations of 3β-hydroxy-5-[4-^{14}C]pregnen-20-one and 3β-hydroxy-5-[4-^{14}C] androsten-17-one with microsomal fraction prepared in EDTA-sucrose.* (A) 3β-hydroxy-5-[4-^{14}C]pregnen-20-one, 100 µg, was incubated for 10 min with 1 ml of microsomal fraction in a final volume of 3 ml; (B) same as in A with the exception that 3 µmoles of NADPH were added; (C) 3β-hydroxy-5-[4-^{14}C]androsten-17-one, 200 µg, was incubated for 10 min with 0.5 ml of microsomal fraction in a final volume of 3 ml; (D) same as in C with the exception that 3 µmoles of NADPH were added. The numbers on the chromatograms represent counts/min. Reference compounds were: (1) 3β,7α-dihydroxy-5-pregnen-20-one; (2) 3β,7β-dihydroxy-5-pregnen-20-one; (3) 3β,16α-dihydroxy-5-pregnen-20-one; (4) 3β-hydroxy-5 pregnene-7,20-dione; (5) 3β-hydroxy-5-pregnen-20-one; (6) 3β,7α-dihydroxy-5-androsten-17-one; (7) 3β,7β-dihydroxy-5-androsten-17-one; (8) 3β-hydroxy-5-androstene-7,17-dione; (9) 3β,16α-dihydroxy-5-androsten-17-one; (10) 3β-hydroxy-5-androsten-17-one; Solvent, 2,2,4-trimethylpentane-iso-amyl acetate—acetone (2:2:1, v/v/v). The chromatoplates were developed three times in the same solvent

assume that one of the hydroxyl groups is located in the 3β-position. The location of the other hydroxyl group can not be completely deduced from the mass spectrum but position 18 is more probable than other positions. A very prominent base peak at M-90 occurs in mass spectra of trimethylsilyl ethers of Δ^5-3β-hydroxysteroids having a hydroxyl group in the C-7 or the C-18 position [31a]. The C-7 position is of course excluded by the chromatographic data (Fig. 5) as well as the crystallization data (Annex, Table 4).

Tables 2 and 3 summarize the effects of cofactors and addition of EDTA to the homogenizing medium on the oxidation of 3β-hydroxy-5-[4-^{14}C]pregnen-20-one and 3β-hydroxy-5-[4-^{14}C]androsten-17-one by the microsomal fraction. The extent of oxidation was the same whether or not EDTA was added to the homogenizing medium. NADPH was several times more efficient as cofactor than NADH. In the presence of microsomal fraction alone or boiled microsomal fraction and NADPH, the extent of oxidation was very small.

Oxidation of 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one by Lipid Peroxides

3β-Hydroxy-5-[4-^{14}C]pregnen-20-one and 3β-hydroxy-5-[4-^{14}C]androsten-17-one were incubated with soybean lipoxidase in the presence of linoleic acid. The pattern of products was essentially the same in both cases and similar to that observed with cholesterol. The predominant product was the corresponding 7-oxo derivative. The extent of formation of the 7-oxo derivative was in both cases about 2% of added substrate.

Effect of Biliary Drainage on Oxidation of Cholesterol, 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one

Table 4 summarizes the effect of biliary drainage on the oxidation of [4-^{14}C]cholesterol, 3β-hydroxy-5-[4-^{14}C]pregnen-20-one and 3β-hydroxy-5-[4-^{14}C]androsten-17-one by rat liver microsomes. In the presence of microsomal fraction from an EDTA-sucrose homogenate of liver from rats with a biliary fistula, the 7α-hydroxylation of cholesterol was about eight times more efficient than was observed with control rats (Table 4, Fig. 7). Biliary drainage resulted

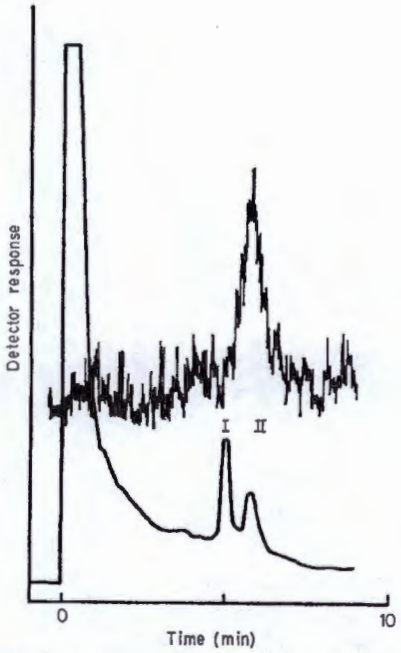

Fig. 6. *Radio-gas chromatogram of material in the zone corresponding to 3β,7β-dihydroxy-5-pregnen-20-one in the chromatogram shown in Fig. 5B.* Unlabeled 3β,7β-dihydroxy-5-pregnen-20-one was added to the labeled material and the mixture was converted to trimethylsilyl ether prior to chromatography. Peak I is the trimethylsilyl ether of 3β,7β-dihydroxy-5-pregnen-20-one. Upper curve, radioactivity tracing; lower curve, mass tracing. Column, 1% SE-30

in a marked increase also in the yield of labeled material with chromatographic properties similar to those of cholestane-3β,5α,6β-triol. Rechromatography of this material showed that the major part was 5-cholestene-3β,7α,12α-triol (Fig. 7C). The rates of 7α-, 7β- + X- and 16α-hydroxylation of 3β-hydroxy-5-[4-^{14}C]pregnen-20-one in the presence of microsomal fraction from rats with a biliary fistula were about 30% of the rates in the presence of microsomal fraction from control rats (Table 4). The rates of 7α-, 7β- and 16α-hydroxylation of 3β-hydroxy-5-[4-^{14}C]androsten-17-one in the presence of microsomal fraction from rats with a biliary fistula were 20—30% of the rates in control experiments.

When microsomal fraction was prepared from a sucrose medium without EDTA, there was no significant difference in rates of formation of 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol, and lipid peroxides between rats with a biliary fistula and control rats (Table 4).

Effect of Phenobarbital Treatment and Carbon Monoxide on Oxidation of Cholesterol, 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one

Treatment with phenobarbital had no significant effect on the 7α-hydroxylation of cholesterol by the microsomal fraction prepared in EDTA-sucrose (Table 5). Carbon monoxide inhibited the reaction (Table 6). In this case, microsomal fraction from rats with a biliary fistula was used. Phenobarbital had no significant effect on the 7α- and 7β- + X-hydroxylations of 3β-hydroxy-5-pregnen-20-one but significantly stimulated the 16α-hydroxylation (Table 5). The 7β- + X-hydroxylations as well as the 16α-hydroxylation were inhibited by carbon monoxide (Table 6). The 7α-hydroxylation tended to be lower in the presence of carbon monoxide but the inhibition was not significant statistically (Table 6). The 16α-hydroxylation of 3β-hydroxy-5-androsten-17-one was not influenced by phenobarbital, whereas the 7α- and 7β-hydroxylations were stimulated three- to four-fold (Table 5). All three hydroxylations were inhibited by carbon monoxide (Table 6).

Table 2. *Metabolism of 3β-hydroxy-5-pregnen-20-one*
The percentages were calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms

Incubation	Homogenizing medium	Conversion of 3β-hydroxy-5-pregnen-20-one into			
		3β,7α-Dihydroxy-5-pregnen-20-one	3β,7β-Dihydroxy-5-pregnen-20-one + 3β,X-dihydroxy-5-pregnen-20-one [a]	3β,16α-Dihydroxy-5-pregnen-20-one	3β-Hydroxy-5-pregnene-7,20-dione
		% conversion of added substrate			
Microsomes	Sucrose	0.1	0.1	0.0	0.2
	EDTA-sucrose	0.4	0.2	0.1	0.6
Microsomes + 3 μmoles of NADPH	Sucrose	1.5	7.4	2.5	2.9
	EDTA-sucrose	2.4	9.6	3.5	1.8
Microsomes + 3 μmoles of NADH	Sucrose	0.5	1.6	0.5	0.4
	EDTA-sucrose	0.6	1.4	0.5	0.3
Boiled microsomes + 3 μmoles of NADPH	Sucrose	0.3	0.2	0.0	0.3
	EDTA-sucrose	0.2	0.2	0.1	0.3

[a] See text.

Table 3. *Metabolism of 3β-hydroxy-5-androsten-17-one*
The percentages were calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms

Incubation	Homogenizing medium	Conversion of 3β-hydroxy-5-androsten-17-one into			
		3β,7α-Dihydroxy-5-androsten-17-one	3β,7β-Dihydroxy-5-androsten-17-one	3β,16α-Dihydroxy-5-androsten-17-one	3β-Hydroxy-5-androstene-7,17-dione
		% conversion of added substrate			
Microsomes	Sucrose	0.2	0.1	0.4	0.4
	EDTA-sucrose	0.2	0.2	0.4	0.4
Microsomes + 3 μmoles of NADPH	Sucrose	6.5	1.1	2.0	1.3
	EDTA-sucrose	7.6	1.2	2.0	1.0
Microsomes + 3 μmoles of NADH	Sucrose	0.5	0.1	0.4	0.4
	EDTA-sucrose	0.5	0.2	0.5	0.4
Boiled microsomes + 3 μmoles of NADPH	Sucrose	0.2	0.1	0.4	0.4
	EDTA-sucrose	0.2	0.1	0.4	0.3

Table 4. *Effect of biliary drainage on oxidation of cholesterol, 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one and on formation of lipid peroxides*
The values listed are the means ± S.D. of experiments with four rats. Abbreviations: EDTA-sucrose, incubations with microsomal fraction prepared in EDTA-sucrose; sucrose, incubations with microsomal fraction prepared in sucrose; control, control rats; bile fistula, rats with biliary drainage for 60 h

Substrate	Reaction	EDTA-sucrose		Sucrose	
		Control	Bile fistula	Control	Bile fistula
		nmoles/mg protein		nmoles/mg protein	
Cholesterol	7α-Hydroxylation	0.14 ± 0.03	1.30 ± 0.18[a]	0.98 ± 0.24	0.87 ± 0.59
	7β-Hydroxylation	0.08 ± 0.04	0.12 ± 0.02	1.32 ± 0.21	0.83 ± 0.67
	Formation of 3β-hydroxy-5-cholesten-7-one	0.13 ± 0.02	0.15 ± 0.03	5.21 ± 1.05	3.63 ± 3.30
				nmoles of malonaldehyde[b]	
Endogenous	Formation of lipid peroxides			40.1 ± 12.7	25.7 ± 12.2
3β-Hydroxy-5-pregnen-20-one	7α-Hydroxylation	1.8 ± 0.6	0.5 ± 0.1[a]		
	7β-Hydroxylation + X-hydroxylation[c]	5.9 ± 1.4	1.9 ± 0.3[a]		
	16α-Hydroxylation	3.0 ± 0.7	0.8 ± 0.2[a]		
3β-Hydroxy-5-androsten-17-one	7α-Hydroxylation	54.3 ± 4.3	13.0 ± 4.2[a]		
	7β-Hydroxylation	7.1 ± 1.0	2.4 ± 0.7[a]		
	16α-Hydroxylation	6.2 ± 1.6	2.3 ± 0.4[a]		

[a] $P < 0.01$ as compared to control.
[b] The figures represent amount formed per ml of microsomal suspension (*cf.* Fig. 2B).
[c] See text.

The formation of 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol and lipid peroxides in the presence of microsomal fraction prepared in sucrose without EDTA was not influenced by phenobarbital treatment (Table 5) or carbon monoxide (Table 6).

DISCUSSION

The results of the present investigation show that there is a correlation between the formation of lipid peroxides and the oxidation of cholesterol by the microsomal fraction of rat liver homogenate. When peroxidation is inhibited, the major product of cholesterol is 5-cholestene-3β,7α-diol. On the other hand, under conditions of NADPH-dependent, enzymatic peroxidation of lipids, cholesterol is oxidized to several products including 3β-hydroxy-5-cholesten-7-one as the predominant product and 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol and cholestane-3β,5α,6β-triol. The same situation prevails when peroxidation is achieved with microsomal fraction and ascorbate. The formation of 3β-hydroxy-5-cholesten-7-one and accompanying products may result from nonenzymatic oxidation of cholesterol by lipid radicals, generated by the lipid peroxidating system. This suggestion is based on the finding that cholesterol is oxidized to

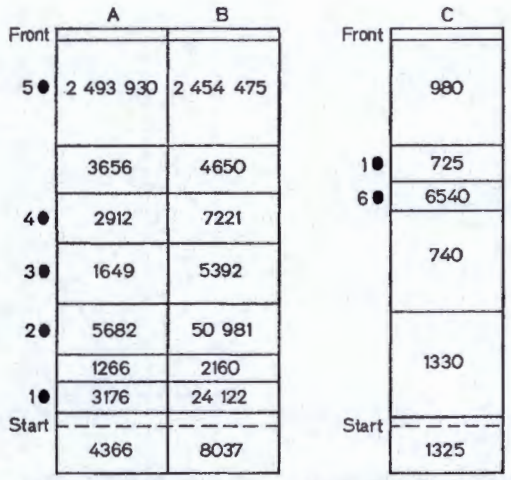

Fig. 7. *Thin-layer chromatograms of extracts of incubations of [4-^{14}C]cholesterol with microsomal fraction from control rat and rat with a biliary fistula.* (A) 3 ml of microsomal fraction from control rat, prepared in EDTA-sucrose, were diluted with buffer to 5 ml and incubated for 15 min with 5 μmoles of NADPH and 15 μg of [4-^{14}C]cholesterol. The numbers on the chromatogram represent counts/min. Reference compounds were: (1) cholestane-3β,5α,6β-triol; (2) 5-cholestene-3β,7α-diol; (3) 5-cholestene-3β,7β-diol; (4) 3β-hydroxy-5-cholesten-7-one; (5) cholesterol. Solvent, benzene—ethyl acetate (2:3, v/v). (B) incubation with microsomal fraction, prepared in EDTA-sucrose, from rat that had had a biliary fistula for two days; incubation conditions, reference compounds and chromatographic conditions as in A. (C) chromatography of material in the zone corresponding to cholestane-3β,5α,6β-triol in chromatogram shown in B. Reference compounds were: (1) cholestane-3β,5α,6β-triol; (6) 5-cholestene-3β,7α,12α-triol. Solvent, ethyl acetate. The chromatoplate was developed three times in the same solvent

the same products by a mixture of soybean lipoxidase and linoleic acid. The correlation between formation of lipid peroxides and oxidation of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one by microsomal fraction was not as apparent as observed for oxidation of cholesterol. A possible explanation is that the enzymatic oxidation in the 7-position of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one is much more efficient than that of cholesterol and makes nonenzymatic oxidation difficult to detect. Both 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one were oxidized in the 7-position by a mixture of soybean lipoxidase and linoleic acid. The pattern of 7-oxidized products formed was similar to that observed for cholesterol.

It has been suggested that the NADPH-dependent, microsomal peroxidation of lipids involves the same electron carriers between NADPH and oxygen as those involved in a number of microsomal hydroxylations [7, 10]. The present results indicate, however, that cytochrome P-450 is not involved in lipid peroxidation since it was not inhibited by carbon monoxide nor stimulated by phenobarbital treatment.

Whether or not oxidation of cholesterol by lipid peroxides is of significance *in vivo* requires further studies. It should be mentioned that the major product of the reaction, 3β-hydroxy-5-cholesten-7-one, is not a presursor of the normally occurring bile acids [6].

The present findings explain the differences in results concerning oxidation of cholesterol by the microsomal fraction between a previous investigation

Table 5. *Effect of phenobarbital treatment on oxidation of cholesterol, 3β-hydroxy-5-pregnen-20-one, and 3β-hydroxy-5-androsten-17-one and on formation of lipid peroxides*

The values listed are the means ± S.D. of experiments with four rats. Abbreviations: EDTA-sucrose, incubations with microsomal fraction prepared in EDTA-sucrose; sucrose, incubations with microsomal fraction prepared in sucrose; control, control rats; phenobarbital, rats treated with daily injections of phenobarbital (100 mg/kg body weight) for 5 days

Substrate	Reaction	EDTA-sucrose		Sucrose	
		Control	Phenobarbital	Control	Phenobarbital
		nmoles/mg protein		nmoles/mg protein	
Cholesterol	7α-Hydroxylation	0.53 ± 0.19	0.29 ± 0.07	0.41 ± 0.14	0.31 ± 0.08
	7β-Hydroxylation	0.11 ± 0.04	0.06 ± 0.02	0.24 ± 0.13	0.14 ± 0.09
	Formation of 3β-hydroxy-5-cholesten-7-one	0.10 ± 0.02	0.10 ± 0.02	0.74 ± 0.45	0.49 ± 0.43
				nmoles of malonaldehyde [b]	
Endogenous	Formation of lipid peroxides			9.3 ± 2.7	15.3 ± 5.2
3β-Hydroxy-5-pregnen-20-one	7α-Hydroxylation	4.8 ± 0.8	7.1 ± 1.1		
	7β-Hydroxylation + X-hydroxylation [c]	19.2 ± 1.5	16.6 ± 2.9		
	16α-Hydroxylation	7.1 ± 0.5	13.8 ± 1.6 [a]		
3β-Hydroxy-5-androsten-17-one	7α-Hydroxylation	34.1 ± 2.2	120.3 ± 8.3 [a]		
	7β-Hydroxylation	6.4 ± 1.3	20.8 ± 2.2 [a]		
	16α-Hydroxylation	8.9 ± 0.9	6.1 ± 0.9		

[a] $P < 0.01$ as compared to control.
[b] The figures represent amount formed per ml microsomal suspension (*cf.* Fig. 2B).
[c] See text.

Table 6. *Effect of carbon monoxide on oxidation of cholesterol, 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one and on formation of lipid peroxides*

The values listed are the means ± S.D. of four incubations. Incubations with cholesterol were carried out with microsomal fraction of liver from rats that had had a bilialy fistula for 48 h. Abbreviations: EDTA-sucrose, incubations with microsomal fraction prepared in EDTA-sucrose; sucrose, incubations with microsomal fraction prepared in sucrose; control, incubations in an atmosphere of 4% oxygen and 96% nitrogen; carbon monoxide, incubations in an atmosphere of 4% oxygen, 56% nitrogen and 40% carbon monoxide

Substrate	Reaction	EDTA-sucrose		Sucrose	
		Control	Carbon monoxide	Control	Carbon monoxide
		nmoles/mg protein		nmoles/mg protein	
Cholesterol	7α-Hydroxylation	2.89 ± 0.52	0.50 ± 0.15[a]	1.00 ± 0.10	1.07 ± 0.12
	7β-Hydroxylation	0.26 ± 0.04	0.11 ± 0.03	1.16 ± 0.28	1.29 ± 0.13
	Formation of 3β-hydroxy-5-cholesten-7one	0.20 ± 0.05	0.18 ± 0.05	3.46 ± 1.00	3.70 ± 0.50
				nmoles of malonaldehyde[b]	
Endogenous	Formation of lipid peroxides			22.5 ± 3.0	29.4 ± 2.3
3β-Hydroxy-5-pregnen-20-one	7α-Hydroxylation	2.9 ± 0.9	1.5 ± 0.2		
	7β-Hydroxylation + X-hydroxylation[c]	14.2 ± 1.6	4.8 ± 0.8[a]		
	16α-Hydroxylation	4.4 ± 0.3	2.5 ± 0.2[a]		
3β-Hydroxy-5-androsten-17-one	7α-Hydroxylation	40.9 ± 3.9	11.4 ± 0.8[a]		
	7β-Hydroxylation	5.7 ± 0.6	2.1 ± 0.8[a]		
	16α-Hydroxylation	11.8 ± 1.4	4.3 ± 0.4[a]		

[a] $P < 0.01$ as compared to control.
[b] The figures represent amount formed per ml microsomal suspension (*cf.* Fig. 2B).
[c] See text.

from this laboratory [6] and investigations by Shefer, Hauser and Mosbach [2] and Boyd, Scholan and Mitton [3]. In the investigation from this laboratory 3β-hydroxy-5-cholesten-7-one was found to be a major product and 5-cholestene-3β,7α-diol a minor product of cholesterol in the presence of microsomal fraction whereas Shefer, Hauser and Mosbach [2] and Boyd, Scholan and Mitton [3] found that 5-cholestene-3β,7α-diol was the predominant product. Shefer, Hauser and Mosbach [2] suggested that the difference in results was due to differences in mode of addition of cholesterol to the incubation mixtures. As is evident from the results of the present investigation, the difference is most probably due to presence or absence of lipid peroxidation in the system. Thus, Shefer, Hauser and Mosbach [2] added EDTA to the homogenizing medium, thereby inhibiting microsomal lipid peroxidation. Boyd, Scholan and Mitton [3] added β-mercaptoethylamine and some sulfhydryl compounds are known to inhibit lipid peroxidation [8, 32]. It might be mentioned in this connection that in short-time incubations with microsomal fraction cholesterol is subjected to true autoxidation only to a very limited extent.

The present investigation also aimed at a further study of the properties of the 7α-hydroxylase system catalyzing the conversion of cholesterol into 5-cholestene-3β,7α-diol. Previous results [33, 34] indicating that this 7α-hydroxylase differs in several respects from other microsomal hydroxylases have been confirmed and expanded. Special interest was paid to a comparison between 7α-hydroxylation of cholesterol and 7α-hydroxylation of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one [35, 36]. Marked differences in the efficiency of the 7α-hydroxylation of the three substrates were observed. The most efficient 7α-hydroxylation was obtained with 3β-hydroxy-5-androsten-17-one and the least efficient with cholesterol. Biliary drainage resulted in a several-fold increase in the 7α-hydroxylation of cholesterol, as has been previously found [2—4, 33, 37]. In contrast, the 7α-hydroxylation of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one was inhibited by biliary drainage. Phenobarbital treatment did not significantly affect 7α-hydroxylation of cholesterol and 3β-hydroxy-5-pregnen-20-one, whereas 7α-hydroxylation of 3β-hydroxy-5-androsten-17-one was stimulated. Carbon monoxide inhibited 7α-hydroxylation of cholesterol and 3β-hydroxy-5-androsten-17-one, indicating the participation of a cytochrome P-450 in these hydroxylations. Cytochrome P-450 may also participate in the 7α-hydroxylation of 3β-hydroxy-5-pregnen-20-one. Further investigations including studies on light reversibility of the inhibition by carbon monoxide are required to establish definitely the participation of cytochrome P-450 in

the 7α-hydroxylations. The apparent explanation for the differences between the three substrates with respect to 7α-hydroxylation is the existence of a specific 7α-hydroxylase for cholesterol and another 7α-hydroxylase(s) for 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one.

The differences between the metabolism of cholesterol and 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one were not limited to the 7α-hydroxylation. Whereas cholesterol was converted practically only to 5-cholestene-3β,7α-diol, which in turn was 12α-hydroxylated to some extent [16], 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one were converted into the corresponding 7-oxo, 7β-, and 16α-hydroxy derivatives [36,38]. 3β-Hydroxy-5-pregnen-20-one was also converted into another, as yet unidentified hydroxylated derivative. It should be mentioned that Hampl and Stárka [39] have reported recently that the microsomal fraction of rat liver homogenate catalyzes the interconversion of 3β,7α-dihydroxy-5-androsten-17-one and 3β,7β-dihydroxy-5-androsten-17-one. The contribution of this reaction to the formation of the two 7-hydroxy derivatives observed in the present investigation cannot be assessed. However, there can be no doubt that there is predominantly an initial 7α-hydroxylation. The amount of 7α-hydroxy derivative formed was several times greater than that of the 7β-hydroxy derivative. Hampl and Stárka [39] found that the equilibrium was towards formation of the 7β-hydroxy derivative and equilibrium was reached not later than after an incubation time of 30 min.

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ANNEXES

The following documents have been deposited at the *Archives Originales du Centre de Documentation du C.N.R.S.* (15 Quai Anatole France, F-75 Paris 7, France) where they may be ordered as microfiche or photocopies. Reference No: A. O.—494.

Annex, Table 1. Crystallization to constant specific radioactivity of products formed in incubations of [4-^{14}C]cholesterol with microsomal fraction prepared in sucrose.

Annex, Table 2. Crystallization to constant specific radioactivity of 5-cholestene-3β,7α-diol formed in incubation of [4-^{14}C]cholesterol with microsomal fraction prepared inEDTA-sucrose.

Annex, Table 3. Crystallization to constant specific radioactivity of products formed in incubation of [4-^{14}C]cholesterol with linoleic acid and soybean lipoxidase.

Annex, Table 4. Crystallization to constant specific radioactivity of products formed in incubation of 3β-hydroxy-5-[4-^{14}C]pregnen-20-one with microsomal fraction prepared in EDTA-sucrose.

Annex, Table 5. Crystallization to constant specific radioactivity of products formed in incubation of 3β-hydroxy-5-[4-^{14}C]androsten-17-one with microsomal fraction prepared in EDTA-sucrose.

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ELSEVIER

Ergosteroids II: Biologically active metabolites and synthetic derivatives of dehydroepiandrosterone

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An improved procedure for the synthesis of 3β-hydroxyandrost-5-ene-7,17-dione, a natural metabolite of dehydroepiandrosterone (DHEA) is described. The synthesis and magnetic resonance spectra of several other related steroids are presented. Feeding dehydroepiandrosterone to rats induces enhanced formation of several liver enzymes among which are mitochondrial sn*-glycerol 3-phosphate dehydrogenase (GPDH) and cytosolic malic enzyme. The induction of these two enzymes, that complete a thermogenic system in rat liver, was used as an assay to search for derivatives of DHEA that might be more active than the parent steroid. Activity is retained in steroids that are reduced to the corresponding 17β-hydroxy derivative, or hydroxylated at 7α or 7β, and is considerably enhanced when the 17-hydroxy or 17-carbonyl steroid is converted to the 7-oxo derivative. Several derivatives of DHEA did not induce the thermogenic enzymes whereas the corresponding 7-oxo compounds did. Both short and long chain acyl esters of DHEA and of 7-oxo-DHEA are active inducers of the liver enzymes when fed to rats. 7-Oxo-DHEA-3-sulfate is as active as 7-oxo-DHEA or its 3-acetyl ester, whereas DHEA-3-sulfate is much less active than DHEA. Among many steroids tested, those possessing a carbonyl group at position 3, a methyl group at 7, a hydroxyl group at positions 1, 2, 4, 11, or 19, or a saturated B ring, with or without a 4-5 double bond, were inactive.* (*Steroids* **63**:158–165, 1998) © *1998 by Elsevier Science Inc.*

Keywords: 7-oxo-dehydroepiandrosterone; DHEA; ergosteroids; acyl esters of 7-oxo-DHEA

Introduction

Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one; hereafter DHEA), the most abundant steroid in human blood, is an intermediate in the biosynthesis of testosterone and estrogens but also exerts several physiological effects independent of the sex hormones.[1] It is an anti-obesity agent for genetically obese[2] and normal[3] animals but does not affect food intake; it is probably not effective in humans.[4] It decreases blood cholesterol concentration in several species,[5–7] lessens the severity of diabetes in genetically predisposed mice,[8] enhances the immune system,[9,10] suppresses tumor development,[11] and improves memory in aged mice.[12]

It is unlikely that these widely different metabolic responses are elicited by a single steroid molecule for which no receptor has been found in liver.[13] Another reason to doubt that DHEA, per se, is responsible is that most of these effects can be observed only when large amounts (0.4–0.6% of the diet) of this steroid are administered. The possible clinical utility of DHEA is limited by its low activity and by its conversion to sex hormones. It can be used by women in only small doses and for limited periods of time, for reasonable doses lead to greatly elevated blood testosterone and dihydrotestosterone concentrations.[14] In female rats DHEA administration leads to excess estrogen and androgen synthesis and the production of polycystic ovaries.[15,16]

To search for possible metabolites of DHEA that might have greater biological activity, greater specificity, and less propensity to form sex hormones, we initiated a program of synthesizing steroids that are derivable from DHEA. The activity of synthesized compounds was monitored by measuring the induction of thermogenic enzymes in rats.[17,18] Hepatic mitochondrial *sn*-glycerol-3-phosphate dehydrogenase and cytosolic malic enzyme function to bypass electron transport from mitochondrial NADH to ubiquinone. The result is a decreased efficiency of coupling phosphorylation to the oxidations of the tricarboxylic acid cycle,[17,19,20] greater heat production, and decreased efficiency of food utilization for growth, fat synthesis or work output.[21]

There are several cytochrome P450 enzymes that hydroxylate steroids at specific positions, and products of such reactions on DHEA have been isolated from tissues, blood and urine of normal and tumor-bearing humans and other mammals.[22–28] Hydroxylations of DHEA in tissue homog-

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enates and cell fractions have been reported from many laboratories.[29–37] 7α-Hydroxy-DHEA[23,26,27] and 7-oxo-DHEA[38–41] are known to occur naturally, mainly as esters. Both 7α- and 7β-hydroxylated DHEA are produced[23,29–33,36] and converted to 7-oxo-DHEA[42–46] by cell fractions.

After testing some commercially available steroids[18,47] we prepared several monohydroxylated derivatives of DHEA [4α, 5α, 7α, 7β, 11β, 16α, 19] and of 5-androstene-3β, 17β-diol [7α, 19]. Only the 7-hydroxy derivatives were active. Because 7-hydroxy-DHEA was fully as active as DHEA[48] and 7-oxo-DHEA was more active[49] we suggested that these compounds might be the first members of a metabolic sequence to more active hormone(s). This hypothesis is consistent with the finding that agents that induce the formation of cytochrome P_{450} 2A1, a steroid 7α-hydroxylase,[37,50] enhance the response of rats to DHEA.[18] None of these 7-substituted structures is convertible to compounds with androgenic or estrogenic activity and they are therefore potentially useful medications for women whereas DHEA is not because, in large doses, it causes masculinization. Because of the ability of these 7-oxygenated steroids to induce thermogenic enzymes that provide a pathway for heat production,[17,20] we have named them ergosteroids.[49]

Experimental

The ability of steroids to induce the formation of liver cytosolic malic enzyme and mitochondrial *sn*-glycerol-3-phosphate dehydrogenase in rats was used as a criterion of DHEA-like activity. Steroids to be assayed were finely pulverized and ground with a small amount of Purina rat chow, then mixed with the requisite amount of chow to give the desired steroid concentration. Records of daily food consumption were kept to ensure that dietary restriction was not influencing the response of the malic enzyme.[51] In some cases the steroid was dissolved in olive oil and injected intraperitoneally.

Male Sprague–Dawley rats (140–160 g) were fed diets containing steroids for 6 days. On Day 7 (at about 200 g), animals were sacrificed, and the large left lobe of the liver was excised and placed in an ice-cold medium containing 250 mM mannitol, 70 mM sucrose, and 3 mM Hepes at pH 7.4. The liver samples were weighed, washed, homogenized and mitochondrial and cytosolic fractions were separated.[52] Mitochondrial glycerol-3-phosphate dehydrogenase was assayed by the procedure of Wernette et al.[53] and cytosolic malic enzyme by that of Hsu and Lardy.[54] Enzyme activities in control rats are expressed as nmol/(min times mg protein). The activities in rats fed steroids are reported as a percent of the activity found in control rats treated similarly but without steroid supplementation. We wish to emphasize that because of variation in the enzyme activity of both control and treated groups from one experiment to another the reported activities are not highly precise—they serve mainly as a guide for further syntheses. Compounds are considered inactive unless they increased the enzyme activity at least 50% above that of the livers of control rats. Maximum increases obtained are about five-fold for glycerophosphate dehydrogenase and ten-fold for malic enzyme. To improve sensitivity, assays were designed with amounts of steroids that gave about half maximal responses; the variability between experiments was found to be relatively greater under these conditions than when maximally effective doses were given.

Dehydroepiandrosterone and some other steroids were purchased from Steraloids, Wilton, New Hampshire; some were from Sigma, St. Louis and Research Plus, Bayonne, New Jersey. Some steroids, other reagents and anhydrous solvents were from Aldrich, Milwaukee, Wisconsin. Many of the compounds tested were prepared by procedures in the literature. We record here the synthesis of only new compounds and improved procedures for some known structures. To save space trivial names are used for well known compounds.

The progress of chemical reactions was monitored by TLC. Melting points (°C) were determined in open capillaries in an electrically heated and stirred Thiele-type bath. They are uncorrected. Nuclear magnetic resonance spectra were acquired with Bruker 200, 300, and 500 MHz spectrometers. Tetramethyl silane (TMS) in $CDCl_3$ was used as internal standard. Chemical shifts are reported on the δ scale with peak multiplicities: d, doublet; dd, double doublet; m, multiplet; q, quartet; s, singlet; and t, triplet. Magnetic resonance data are included for some known compounds where such data were not previously reported.

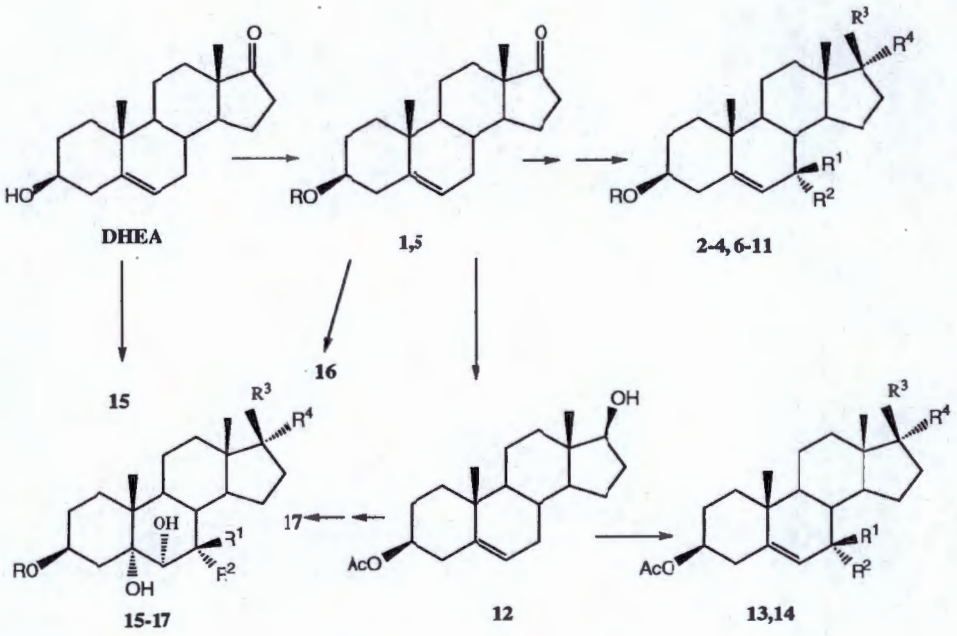
Syntheses

3β,7α-Dihydroxyandrost-5-en-17-one (2)

This compound was initially synthesized according to the method of Starka.[55] However, the low yields and lack of generality of that method prompted a search for another route to the 7-hydroxy steroids. In the method described below DHEA acetate is subjected to allylic bromination by a procedure developed by Confalone et al.[56] to prepare 7α derivatives of cholesterol. The mixture of 7α- and β-bromo DHEA acetate that is formed may be isomerized to predominantly the 7α-bromo compound and then subjected to a stereospecific oxidative debromination in the presence of acetic acid and silver acetate.

Ten g of DHEA acetate (**1**; 0.03 mol) and 13.6 g of $NaHCO_3$ (0.016 mol) were stirred with 1 L hexane (b.p. 69–71°C) and heated to reflux under N_2. Dibromantin (1,3-dibromo-5,5-dimethylhydantoin (6.11 g; 0.021 mol) was added and the heating continued for 30 min. The mixture was then cooled to room temperature and filtered. The residue was extracted with 50 mL of CH_2Cl_2 and the combined organic phases were rotary evaporated to near dryness at less than 35°C. The creamy white product is unstable to storage and should be used immediately.

The 7α- and 7β-bromo derivatives were dissolved in 80 mL CH_2Cl_2, and 8 g anhydrous LiBr in 320 mL ice-cold acetone was added. The mixture was shielded from light and stirred on ice for 3 h during which time it was converted predominantly to the 7α-bromo enantiomer. Silver acetate (26 g) suspended in 320 mL CH_2Cl_2 and 80 mL of glacial acetic acid was stirred for 20 min at room temperature and then poured into the solution of 7-bromo-DHEA acetate. The mixture darkened as it was stirred for 30 min. The mixture was filtered and the solids were rinsed with CH_2Cl_2. The combined filtrate was extracted 3 times with 1 L H_2O, residual acid was removed with 5% $NaHCO_3$, extracted twice with water and rotary evaporated to dryness. The ester was dissolved in 500 mL methanol and heated to reflux under N_2 with stirring; 250 mL of 5% Na_2CO_3 were added and refluxing continued for 45 min. The methanol was evaporated and the solution carefully neutralized with acetic acid. Extraction into CH_2Cl_2 was followed by evaporation and azeotropic drying with absolute ethanol and twice with acetone. The solid 7α-hydroxy DHEA was dissolved in a minimum amount of warm acetone and hexane was added to incipient turbidity. Crystals form at room temperature (m.p. 187–189°C) and a second crop may be obtained from the filtrate by storing at lower temperature. Depending on the scale of the reaction the yield is from 50 to 80% based on DHEA acetate. The recrystallized compound melted at 192–193° (lit.[29] 182–183°C). In the event that crystallization does not occur the solution may be evaporated, dried as above and the product purified by chroma-



Scheme 1

tography on silica gel (petroleum ether:AcOEt 2.5:1, 1.5:1, then 1:1). An alternative route to either 3β-acetoxy-7α—or 3β-acetoxy-7β-hydroxyandrost-5-en-17-one has been reported recently from this laboratory.[57]

3β-Acetoxyandrost-5-ene-7,17-dione (3)

The synthesis of the 7-oxo-DHEA acetate used in the bioassays described in this paper was based on a patent by Forischer et al.[58] All starting material should be thoroughly purified and free of any acidic or basic impurities; if not, reaction time increases and the yield of product decreases.

In a two-necked flask *N*-hydroxyphthalimide (10.86 g, 0.066 mol) was taken up in a mixture of acetone and ethyl acetate (1:1, 200 mL). 3β-Acetoxyandrost-5-en-17-one (**1**; 22.0 g, 0.066 mol) and azobis (cyclohexane-carbonitrile, 1.1 g) were added in succession. Subsequently, a weak stream of compressed air was passed into the mixture which was allowed to reflux for 9 h. After completion of the reaction (as monitored by TLC: ethyl acetate: hexane 1:1.5) the mixture was evaporated to dryness and the resultant mass taken up in 150 mL of toluene. Stirring at 50°C for 30 min followed by cooling at room temperature gave a white crystalline precipitate of *N*-hydroxyphthalimide which was filtered off, washed with two 10 mL portions of toluene, and dried under suction. Thereby, 10 g (90%) of *N*-hydroxyphthalimide was recovered and may be reused.

The organic layer was washed thoroughly with saturated sodium bicarbonate solution until the aqueous layer was colorless, then with water. Toluene is distilled off under suction and the residue was heated to dissolve in 130 mL of methanol. On cooling the 7-oxo compound crystallized. It was collected on a filter, washed with cold methanol and dried under vacuum. 3β-Acetoxyandrost-5-ene-7,17-dione (**3**) was thus obtained as an off white colored compound (13.2 g, 56.7% yield). It was further purified by recrystallization from acetone-hexane, which gave 11.5 g of white crystalline product (50% yield), with a melting point of 185–187°C.

The mother liquor, when chromatographed on silica gel (eluent–ethyl acetate/hexane 1:5) yielded another 5.0 g of pure 3β-acetoxyandrost-5-ene-7,17-dione (combined yield 72%). U.V.: max 235 nM, E = 13,700 (c = 0.032 g/L, ethanol). Infra red: 1741, 1724, 1669, 1240 cm. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.78 (1H, s, 6-H), 4.74 (1H, m, 3α-H), 2.08 (3-H, s, CH_3-acetate), 1.24 (s, 3H, 19-CH_3), 0.90 (s, 3H, 18-CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 221 (C-17), 200 (C-7), 170 (C=O, acetate), 164.5 (C-5), 126.3 (C-6), 71.8 (C-3), and 50, 47.7, 45.8, 44.3, 38.4, 37.8, 35.9, 35.4, 30.7, 27.2, 24.0, 21.0, 20.5, 17.3, 13.6.

3β-Hydroxyandrost-5-ene-7,17-dione (4)

3β-Hydroxyandrost-5-ene-7,17-dione (**4**) was prepared from the acetate (**3**) by saponification with Na_2CO_3 in methanol M.p. 236–239°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.74 (d, J = 1.47 Hz, 6-H), 3.69 (m, 3α-H), 1.23 (s, 19-CH_3), 0.90 (s, 18-CH_3). ^{13}C NMR (200 MHz, $CDCl_3$): δ 219.8 (C-17), 200.7 (C-7), 166.2 (C-5), 125.7 (C-6), 70.2 (C-3), 50.3, 47.8, 45.9, 44.4, 41.9, 38.4, 36.4, 35.5, 31.14, 30.8, 24.1, 20.6, 17.4, 13.71.

Acyl esters of 3β-hydroxyandrost-5-ene-7,17-dione and related steroids (5–13)

Propionyl, butyryl, and long-chain fatty acyl esters of DHEA have been described in the literature and some are items of commerce. Isobutyryl DHEA and propionyl, butyryl, and isobutyryl esters of 7-oxo-DHEA were prepared by reacting the parent steroid with a 10% molar excess of the corresponding acid anhydride in anhydrous pyridine; acid chlorides were used for preparing the long chain esters. When acylation was completed the mixture was poured into ice-water, carefully neutralized with HCl, collected by filtration, washed with water, dried and crystallized from ethanol or acetone-hexane. A second crop was usually obtained by storing the filtrate at 4°C. Some esters of 7-oxo-DHEA were also prepared by oxidizing the corresponding ester of DHEA.

3β-isoButyroxyandrost-5-en-17-one (5). M.p. 187–188°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.4 (1H, d, J = 5.0 Hz, 6-H), 4.59 (1H, m, 3α-H), 1.18 (3H, s, CH_3), 1.14 (3H, s, CH_3), 1.05 (3H, s, 19-CH_3), 0.89 (3H, s, 18-CH_3).

3β-Butyroxyandrost-5-ene-7,17-dione (6). M.p. 219–220°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.76 (1H, d, J = 1.4 Hz, 6-H), 4.75 (1H, m, 3α-H), 1.24 (3H, s, 19-CH_3), 0.96 (3H, t, J = 7.4 Hz, CH_3), 0.90 (3H, s, 18-CH_3).

3β-isoButyroxyandrost-5-ene-7,17-dione (7). M.p. 185–186°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.78 (1H, s, 6-H), 4.74 (1H, m, 3α-H), 1.22 (3H, s, 19-CH_3), 1.18 (6H, s, $(CH_3)_2$), 0.9 (3H, s, 18-CH_3).

3β-Dodecanoyloxyandrost-5-ene-7,17-dione (8). M.p. 90–91°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.77 (1H, s, 6H), 4.73 (1H, m, 3α-H), 1.24 (s, 19-CH_3), 0.9 (s, 18-CH_3).

3β-Hexadecanoyloxyandrost-5-ene-7,17-dione (9). M.p. 87–88°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.76 (1H, s, 6H), 4.73 (1H, m, 3α-H), 1.2 (s, 19-CH_3), 0.9 (s, 18-CH_3).

3β-Octadecanoyloxyandrost-5-ene-7,17-dione (10). M.p. 87–88°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.76 (1H, s, J = 1.4 Hz, 6H), 4.74 (1H, m, 3α-H), 0.90 (3H, s, 18-CH_3).

3β-Hemisuccinoyloxyandrost-5-ene-7,17-dione (11). M.p. 265–265.5°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.76 (1H, s, J = 1.6 Hz, 6-H), 4.76 (1H, m, 3α-H), 1.24 (3H, s, 19-CH_3), 0.9 (3H, s, 18-CH_3).

3β-Acetoxyandrost-5-en-17β-octadecanoate (13). To 0.85 g of 3β-acetoxyandrost-5-en-17β-ol (**12**) in 10 mL dry pyridine, 1 g of stearoyl chloride was added at room temperature. Ninety percent of the starting steroid had reacted within 30 min and none could be detected the following day when the reaction mixture was worked up as described above. The crystalline product, 1.394 g (91%) melted at 82–83°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.37 (1H, s, J = 5.4 Hz, 6H), 4.6 (2H, m, 3α-H + 17α-H), 2.04 (3H, s, $COCH_3$), 1.03 (3H, s, 19-CH_3), 0.80 (3H, s, 18-CH_3).

Table 1 Enzyme Induction by Esters of DHEA and 7-Oxo-DHEA

Compound	Concentration in diet	No. of rats	GPDH % of control	ME % of control
DHEA	0.05	5	232	295
DHEA-acetate	0.05	13	210	276
7-Oxo-DHEA-acetate	0.052	13	238	378
DHEA-propionate	0.052	6	240	292
7-Oxo-DHEA-propionate	0.054	6	250	379
DHEA	0.05	3	202	311
DHEA-acetate	0.057	5	263	351
DHEA-butyrate	0.062	2	175	196
7-Oxo-DHEA-butyrate	0.013	3	119	196
7-Oxo-DHEA-butyrate	0.065	4	291	459
DHEA-isobutyrate	0.062	6	97	125
7-Oxo-DHEA-isobutyrate	0.064	6	208	285
DHEA-acetate	0.05	6	152	261
DHEA-laurate	0.08	6	159	228
7-Oxo-DHEA-laurate	0.08	3	158	221
DHEA-palmitate	0.08	5	194	235
7-Oxo-DHEA-palmitate	0.05	2	146	154
7-Oxo-DHEA-palmitate	0.09	5	238	292
7-Oxo-DHEA-acetate	0.02	2	209	396
DHEA-stearate	0.096	2	202	205
7-Oxo-DHEA-stearate	0.036	2	151	228
7-Oxo-DHEA-sulfate	0.067	3	305	356
3β-Acetoxy-A-17β-stearate	0.035	2	120	143
3β-Acetoxy-17β-stearoyl-A-7-one	0.036	2	134	165
DHEA-acetate	0.05	8	220	346
DHEA-hemisuccinate	0.06	4	205	353
7-Oxo-DHEA-acetate	0.05	2	268	429
7-Oxo-DHEA-hemisuccinate	0.012	2	172	177
7-Oxo-DHEA-hemisuccinate	0.03	2	206	213
7-Oxo-DHEA-hemisuccinate	0.06	6	333	488

In each assay, enzyme activities of rats fed the new esters for six days were compared with rats fed DHEA or DHEA-acetate and with non-supplemented controls. All acyl groups are at the 3β position unless designated at 17β. GPDH, sn glycerol 3-phosphate dehydrogenase; ME, malic enzyme. In this table -A- is an abbreviation for androst-5-ene.

3β-Acetoxy-17β-octadecanoyloxyandrost-5-en-7-one (14)

M.p. 87–90°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.71 (1H, s, J = 1.0 Hz, 6-H), 4.71 (1H, m, 3α-H), 4.64 (1H, m, 17α-H), 2.05 (3H, s, $COCH_3$), 1.22 (3H, s, 19-CH_3), 0.81 (3H, s, 18-CH_3).

3β,5α,6α-Trihydroxyandrostan-17-one (15)[59]

M.p. 239–242°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 4.05 (m, 3α-H), 3.73, 3.67 (dd, J = 5.4, 11.2 Hz, 6β-H), 0.96 (s, 19-CH_3), 0.84 (18-CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 210 (C-17), 70.2, 67.1 (C-3,6), 50.9, 44.4, 33.3 (C-methines), 38.4, 35.8, 33.8, 31.5, 31.1, 30.6, 21.7, 20.4 (C-methylenes), 15.5, 13.8 (C-methyls).

3β-Acetoxy-5α,6α-dihydroxyandrostane-7,17-dione (16)

A solution of 1.15 g of 3β-acetoxyandrost-5-ene-7,17-dione (**3**) in 10 mL of pyridine was treated with 0.98 g of OsO_4 in 15 mL of pyridine and stirred for 2.5 h at room temperature. A solution of 1.8 g of sodium bisulfite in 30 mL of water and 20 mL of pyridine was added to cleave the osmate ester. After stirring for 20 min the mixture was extracted repeatedly with dichloromethane (100, 50, 50, 25 mL) which was washed with saline and water, and dried over $MgSO_4$. When concentrated under reduced pressure the product crystallized, was collected by filtration and dried in vacuo. Yield 930 mg. M.p. 238–240°C. The mother liquor yielded 191 mg (m.p. 237–238°C) for an overall yield of 88%. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.05 (m, 3α-H), 4.12 (d, J = 1.00 Hz, 6β-H), 2.03 (s, $OCOCH_3$), 1.32 (s, 19-CH_3), 0.88 (s, 18-CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 219.6 (C-17), 209.3 (C-7), 170.5 (C=O acetate), 81.3 (C-5), 77.1, 70.0 (C-3,6), 47.1, 46.7, 44.3 (C-methines), 35.5, 34.9, 30.7, 30.6, 26.5, 22.6, 21.0 (C-methylenes), 21.3, 16.0, 13.8 (methyls).

3β-17β-Diacetoxy-5α,6α-dihydroxyandrostan-7-one (17)

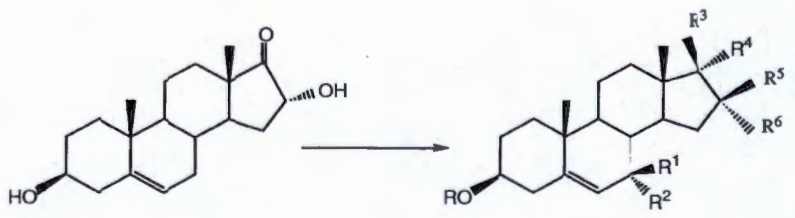
This compound, prepared as for the diketone above, melted at 159–160°C when recrystallized. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.06 (m, 3α-H), 4.63 (t, J = 8.3 Hz, 17α-H), 4.08 (d, J = 2.9 Hz,

Table 2 Steroids that Induce the Synthesis of Mitochondrial Glycerophosphate Dehydrogenase and Cytosolic Malic Enzyme in Rats' Livers

Steroid	Concentration in diet	No. of rats	GDPH	ME
			% of control	
3β-ol-A-17-one (DHEA)	0.01	8	124	107
	0.025	3	157	178
	0.05	135	253 ± 56	327 ± 76
	0.1	68	295 ± 68	428 ± 86
	0.2	4	285	645
3β-acetoxy-A-17-one	0.023	3	148	285
	0.057	42	241 ± 55	329 ± 75
	0.1	9	285 ± 64	455 ± 105
3β-ol-A-7,17-dione (7-oxo-DHEA)	0.0105	6	168 ± 17	244 ± 61
	0.026	5	290 ± 32	499 ± 47
	0.052	11	341 ± 88	507 ± 71
	0.105	6	366	704
3β-acetoxy-A-7,17-dione	0.02–0.03	9	215 ± 66	341 ± 85
	0.052–0.06	19	304 ± 107	392 ± 40
	0.1–0.12	15	270 ± 59	463 ± 113
3β,7α-diol-A-17-one	0.033	2	308	374
	0.05	2	292	423
3β-acetoxy-7β-ol-A-17-one	0.05	3	219	339
A-3β,17β-diol	0.05	4	234	261
	0.2	3	275	494[a]
3β,17β-diol-A-7-one	0.01	8	216	219
	0.05	5	378	416
	0.1	6	231	652
3β-acetoxy-A-17β-ol	0.058	2	222	338
3β-acetoxy-17β-ol-A-7-one	0.13	3	232	452
3β,17β-diacetoxy-A	0.065	8	114	131
3β,17β-diacetoxy-A-7-one	0.068	9	290	274
3β,16α-diol-A-17-one	0.05	5	99	135
A-3β,7α,17β-triol	0.1	2	227	611
3β,16α,17α-triacetoxy-A	0.075	3	145	107
3β,16α,17α-triacetoxy-A-7-one	0.078	3	198	164
3β,16α,17α-triol-A-7-one	0.056	3	102	227
3β,16α-diacetoxy-7α-ol-A-17-one	0.05	2	246	270
3β,16β-diacetoxy-7β-ol-A-17-one	0.05	2	228	507
3β,16β-diacetoxy-7α-ol-A-17-one	0.05	5	242	340
3β-propionoxy-7α-F-A-17-one	30 mg/day times 3	3	213	164
3β-acetoxy-7α-F-A-17-one	30 mg/day times 3	3	157	290
3β-acetoxy-7β-F-A-17-one	30 mg/day times 3	3	167	258
3β-acetoxy-7α-F-A-17-one	20 mg/day times 3	3	185	270
3β-acetoxy-7β-F-A-17-one	20 mg/day times 3	3	224	231

In this table, -A- is an abbreviation for androst-5-en. Enzyme activity in the livers of rats fed the stock diet without supplementation is termed 100%.
[a]Data from Su and Lardy (18).

6β-H), 3.83 (d, OH), 2.04, 2.02 (2s, 3,17-acetate), 1.31 (s, 19-CH_3), 0.80 (s, 18-CH_3). ^{13}C NMR (300 MHz, $CDCl_3$): δ 209.5 (C-7), 171.05, 170.48 (C═O, acetates), 81.27 (C-5), 81.78, 77.02, 70.16, (C-3,6,17), 47.27, 46.86, 43.48 (C-8,9,14), 35.74, 34.81, 30.7, 27.48, 26.46, 24.3, 21.22 (C-1,2,4,11,12,15,16), 21.31, 21.1 (acetate-CH_3), 15.96, 12.08 (C-18,19).



18-21

Scheme 2

Table 3 Compounds that Do Not Induce Mitochondrial Glycerophosphate Dehydrogenase or Cytosolic Malic Enzyme in Rats' Livers

Androst-4-ene-3β,17β-diol; androst-4-ene-3,17-dione; androst-4-ene-3,11,17-trione (0.052); 4-hydroxyandrost-4-ene-3,17-dione (0.015); 11β-hydroxyandrost-4-ene-3,17-dione (0.05); 19-hydroxyandrost-4-ene-3,17-dione (0.024); 7α-hydroxytestosterone; 17α-ethynyltestosterone; 17-hydroxyandrosta-4,6-dien-3-one; 3β-acetoxy-5α-ol-androst-6-en-17-one (0.05).

Androst-5-en-17-one; androst-5-ene-7,17-dione; 3β-hydroxy-7-methylandrost-5-en-17-one; 4β-hydroxy-DHEA; 16α-hydroxy-DHEA; 16α-bromo-DHEA; 19-ol-DHEA; androst-5-ene-3β,7α,19-triol; 1α,3β-diacetoxyandrost-5-ene-7,17-dione; 2α,3β-diacetoxyandrost-6-en-17-one; androst-5-ene-3β,16α-diol; 3β,17β-dihydroxyandrost-5-en-11-one (0.053); 3β-acetoxy-5α-hydroxyandrost-6-en-17-one; androst-5-ene-3β,19,17β-triol; 2β,3β,17β-triacetoxyandrost-5-en-7-one; 3β,7α-dihydroxy-16β-acetoxyandrost-5-en-17-one (0.05); 3β-hydroxyandrost-5-ene-17-carboxylic acid; 3β-acetoxyandrosta-1,5-diene-7,17-dione; androsta-3,5-diene-7,17-dione (0.05); 17β-hydroxyandrosta-3,5-dien-7-one; 3β-hydroxyandrosta-5,7-dien-17-one; androsta-5,16-dien-3β-ol, and its 3-acetate.

11β-hydroxyandrosterone; 11-oxo-androsterone (0.04); 3β-acetoxy-5α-androstane-7α,17β-diol (0.01); 3β-acetoxy-5α,6α-dihydroxyandrostane-7,17-dione; 3β,5α,6α-trihydroxyandrostan-17-one (0.056); 3β,17β-diacetoxy-5α,6α-dihydroxyandrostan-7-one; 3β,5α,6α,16α,17α-pentacetoxyandrostane; 2α,3α-dihydroxy-5α-androstane-7,17-dione; 3β,5α,6β-trihydroxyandrostan-17-one; 17β-hydroxy-5α-androstane-3,7-dione (0.027).

5β-androstane-3α,17β-diol (0.067); 5β-androstane-3β,17β-diol (0.077), and its 3-acetate (0.05); 3β-hydroxy-5β-androstan-17-one (0.077) and its 3-acetate (0.077); 5β-androstane-3,17-dione (0.077); 5β-androstane-3α,7α,17β-triol (0.043); 7α,17β-dihydroxy-5β-androstan-3-one-(0.05); 3α,7α,16α-trihydroxy-5β-androstan-17-one (0.08), and its 16-acetate (0.05); 7α-hydroxy-16α-acetoxy-5β-androstane-3,17-dione (0.05).

7-hydroxyestrone (0.01); androsta-1,4,6-triene-3,7-dione; 17α-ethyl-17β-ol-19-nor androst-4-en-3-one; 3α,6α-dihydroxy-5β-pregnan-20-one (0.05); 3α-hydroxy-5β-pregnan-20-one (0.05); 16α-hydroxyprogesterone (0.06); pregnenolone; 7α-hydroxypregnenolone; 3β-acetoxypregnene-7,20-dione (0.06); 3β,17α-diacetoxypregnen-7,20-dione.

These steroids were fed at 0.1 or 0.2% of the diet unless a lower concentration is indicated. For comparative purposes, some additional inactive compounds are reported in Tables 1 and 2.

3β,16α-diacetoxyandrost-5-en-17-one (18)

Four g of 3β,16α-dihydroxyandrost-5-en-17-one[60] in 24 mL Ac_2O and 16 mL pyridine was stirred for 12 h at room temperature. After removal of solvents in vacuo, the residue was purified over silica gel ($CHCl_3$: Me_2CO = 60:1) to yield compound **18** (3 g, 67%) as a white solid which was crystallized from AcOEt/pet ether. M.p. 165–167°C. ^{1}HMR (300 MHz, $CDCl_3$) δ 5.20 (m, 2 H, 6-H and 16-H), 4.60 (m, 1 H, 3-H), 2.09 (s, $OCOCH_3$), 2.02 (s, $OCOCH_3$), 1.06 (s, CH_3), 1.01 (s, CH_3).

3β,16α-diacetoxy-7-α-hydroxyandrost-5-en-17-one (19) and 3β,7α,16α-triacetoxyandrost-5-en-17-one (20)

Compound **18** (3 g) was brominated, treated with LiBr and subsequently with AgOAc as in the procedure for compound **2** except that toluene was the solvent to which the LiBr solution was added. Thirty min after the addition of AgOAc, solids were removed by filtration and washed; the filtrate was concentrated and 300 mL H_2O was added to the residue. The pH was brought to neutral with $NaHCO_3$ and the solution was extracted with AcOEt (150 mL times 5). The combined organic solution was washed with brine, dried and concentrated to dryness. The crude product was purified over silica gel (AcOEt:pet ether = 1:3, 1:2, and then 1:1) to yield 1.5 g of diacetate **19** (48%) and 700 mg of triacetate **20** (20%). Both compounds were crystallized from Et_2O.

Compound **19.** M.p. 155–158°C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.64 (d, 1H, J = 4 Hz, 6-H), 5.44 (d, 1H, J = 5 Hz, 16-H), 4.63 (m, 1H, 3-H), 3.92 (m, 1H, 7-H), 2.15 (s, $OCOCH_3$), 2.04 (s, $OCOCH_3$), 1.02 (s, CH_3), 0.97 (s, CH_3).

Compound **20.** M.p. 170–172°C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.61 (d, 1H, J = 2 Hz, 6-H), 5.40 (d, 1H, J = 4 Hz, 16-H), 5.02 (dd, 1H, J = 2 Hz, 7-H), 4.70 (m, 1H, 3-H), 2.10 (s, $OCOCH_3$), 2.02 (s, $OCOCH_3$), 1.02 (s, CH_3), 0.98 (s, CH_3).

3β,7α,17β-Trihydroxyandrost-5-en-16-one (21)

Diacetate **19** (400 mg) was stirred with 342 mg K_2CO_3 in 25 mL MeOH at room temperature for 2 h. After removal of insoluble salt the filtrate was concentrated in vacuo and the residue was purified over silica gel. The white solid was crystallized from MeOH/Et_2O to yield triol **20** (180 mg, 56%. M.p. > 230°C. ^{1}H NMR (300 MHz, DMSO) δ 5.42 (d, 1H, J = 2 Hz, 6-H), 5.30 (br s, 1H, OH, D_2O exch.), 4.68 (br s, 1H, OH, D_2O exch.), 4.26 (d, 1H, J = 2 Hz, OH, D_2O exch.), 3.64 (s, 1 H, 17-H), 3.60 (br s, 1H, 7-H), 3.32 (m, 3-H), 0.92 (s, CH_3), 0.61 (s, CH_3).

Results and discussion

Many therapeutically useful steroids are administered as esters and the widespread distribution of esterase activity[61] in mammalian tissues ensures the cellular availability of the free steroid. Several aliphatic esters of DHEA and of its 7-oxo analog were synthesized to ascertain their possible utility in therapy and to provide standards for metabolic studies; their activity as inducers of rat liver glycerol-3-phosphate dehydrogenase and malic enzyme is shown in Table 1. Unexpected selective hydrolyses appear to be involved in the availability of some of these steroids. Among the monoesters prepared and tested all were active except the isobutyryl derivative of DHEA; however, isobutyryl-7-oxo-DHEA was fully active. Other examples of inactive esters matched by active 7-oxo derivatives are presented in Table 2 and are discussed below. The sulfate ester of 7-oxo-DHEA, which is found in humans,[40] was approximately as active as equimolar amounts of DHEA-acetate whereas DHEA sulfate was poorly active orally and especially when injected intraperitoneally.[18]

Long chain fatty acyl derivatives of steroids occur naturally[62–66] and some may have special functions.[64] The weak enzyme induction by 17β-stearoyl derivatives of 3β-acetoxy-androstenediol and the corresponding 7-oxo compound indicates that these esters are not readily hydrolyzed by tissue esterases. Hochberg et al. have observed that fatty acyl esters of testosterone[64] and estrogens[65] also have prolonged half lives in rats.

The finding that oxygenated derivatives of dehydroepiandrosterone occur in the urine of normal subjects and of patients with adrenal tumors[22,23] led Okada et al.[23] to pos-

tulate that the parent C_{19} ketosteroid might be converted to other active hormones as well as to testosterone and estrogens. Mammalian tissue preparations hydroxylate DHEA to produce 7α, 7β, and 16α-hydroxy derivatives and 7-oxo-DHEA.[29–37] Such oxygenated analogs of DHEA had apparently not been tested for biological activity in animals until recently.[18,47–49] The fact that there is no known receptor for DHEA and that relatively massive amounts (ca. 0.5% of the diet; or ca. 500 mg/kg body wt. per day) are required to elicit its antiobesity effect in mice and rats indicates that DHEA is not an active hormone but only a precursor of an active anti-obesity factor. We felt that some of the known metabolites of DHEA might be intermediates in the biological conversion of DHEA to more active hormones that have yet to be discovered. We therefore tested these known metabolites and synthesized additional compounds that might be produced from DHEA in mammalian tissues or that might shed light on structure/activity relations.

A comparison of the structures of compounds that induce the formation of liver mitochondrial *sn*-glycerol-3-phosphate dehydrogenase and cytosolic malic enzyme in rats (Table 2) with those that do not (Table 3) is informative. The conversion of precursors of DHEA to active steroids in the brain and testes of rats apparently does not occur rapidly enough to provide DHEA to the liver for feeding pregnenolone, 7α-hydroxypregnenolone, or 7-oxo-17α-hydroxypregnenolone had no influence on the activity of these enzymes in liver. Introduction of hydroxyl groups into DHEA or 7-oxo-DHEA at position 1, 2, 4, 11, or 19 abolished the activity as did saturating the B ring or moving the double bond from 5-6 to 4-5. Introducing a second double bond at 1-2 or 7-8 abolished activity as did oxidizing the 3β-hydroxyl group to form a ketone. The 16α-hydroxy derivative of DHEA is produced in normal and tumor bearing humans. It did not induce the formation of glycerol-3-phosphate dehydrogenase and had only a minor influence on malic enzyme (Table 2); likewise the 16α-hydroxy derivative of androstenediol assayed as the triacetate was inactive but the corresponding 7-oxo derivatives of both were active. The diacetyl ester of androstenediol was inactive but 7-oxo-androstenediol diacetate was as effective as free androstenediol (Table 2).

Both 7α- and 7β-hydroxy DHEA induce the formation of the thermogenic enzymes and both are known to be oxidized to 7-oxo DHEA in tissue preparations.[29,32,44] The 7-oxo derivatives of both DHEA and androstenediol are the most active compounds tested. They effect good responses when fed at only 0.01% of the diet whereas DHEA at that concentration fails to raise the enzyme activity above our arbitrary score of 150%. To eliminate the inter-experiment variation in response to the steroids a dose-comparison assay was conducted in a single 27-rat experiment (the assay results are included in Table 2). Within that single experiment, over the range 0.01 to 0.1% of the diet, 7-oxo-DHEA was 2.5 times as active as DHEA. Some 60 steroids that do not induce the thermogenic enzymes are recorded in Table 3. Selected members of this group are being tested for other activities.

In view of the fact that rat liver does not contain the 17α-hydroxylase that participates in the conversion of pregnenolone to DHEA, and that there is not a significant amount of the latter in rat blood, it is astonishing that the rat responds so readily to administered DHEA, androstene diol and their 7-oxygenated derivatives. Because pregnenolone and 17α-hydroxy pregnenolone did not enhance the liver enzymes, it does not seem likely that small amounts of DHEA produced in brain and testes or ovaries are rapidly converted to an active steroid to which liver is responsive.

It seems likely that the 7-hydroxy derivatives of DHEA, and 7-oxo-DHEA are on an enzyme-catalyzed pathway to one or more active hormones that are the ultimate effectors of the several metabolic responses to DHEA.

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Effects of Aromatase Inhibition in Elderly Men with Low or Borderline-Low Serum Testosterone Levels

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As men age, serum testosterone levels decrease, a factor that may contribute to some aspects of age-related physiological deterioration. Although androgen replacement has been shown to have beneficial effects in frankly hypogonadal men, its use in elderly men with borderline hypogonadism is controversial. Furthermore, current testosterone replacement methods have important limitations.

We investigated the ability of the orally administered aromatase inhibitor, anastrozole, to increase endogenous testosterone production in 37 elderly men (aged 62–74 yr) with screening serum testosterone levels less than 350 ng/dl. Subjects were randomized in a double-blind fashion to the following 12-wk oral regimens: group 1: anastrozole 1 mg daily (n = 12); group 2: anastrozole 1 mg twice weekly (n = 11); and group 3: placebo daily (n = 14). Hormone levels, quality of life (MOS Short-Form Health Survey), sexual function (International Index of Erectile Function), benign prostate hyperplasia severity (American Urological Association Symptom Index Score), prostate-specific antigen, and measures of safety were compared among groups.

Mean ± SD bioavailable testosterone increased from 99 ± 31 to 207 ± 65 ng/dl in group 1 and from 115 ± 37 to 178 ± 55 ng/dl in group 2 ($P < 0.001$ *vs.* placebo for both groups and $P = 0.054$ group 1 *vs.* group 2). Total testosterone levels increased from 343 ± 61 to 572 ± 139 ng/dl in group 1 and from 397 ± 106 to 520 ± 91 ng/dl in group 2 ($P < 0.001$ *vs.* placebo for both groups and $P = 0.012$ group 1 *vs.* group 2). Serum estradiol levels decreased from 26 ± 8 to 17 ± 6 pg/ml in group 1 and from 27 ± 8 to 17 ± 5 pg/ml in group 2 ($P < 0.001$ *vs.* placebo for both groups and P = NS group 1 *vs.* group 2). Serum LH levels increased from 5.1 ± 4.8 to 7.9 ± 6.5 U/liter and from 4.1 ± 1.6 to 7.2 ± 2.8 U/liter in groups 1 and 2, respectively ($P = 0.007$ group 1 *vs.* placebo, $P = 0.003$ group 2 *vs.* placebo, and P = NS group 1 *vs.* group 2). Scores for hematocrit, MOS Short-Form Health Survey, International Index of Erectile Function, and American Urological Association Symptom Index Score did not change. Serum prostate-specific antigen levels increased in group 2 only (1.7 ± 1.0 to 2.2 ± 1.5 ng/ml, $P = 0.031$, compared with placebo).

These data demonstrate that aromatase inhibition increases serum bioavailable and total testosterone levels to the youthful normal range in older men with mild hypogonadism. Serum estradiol levels decrease modestly but remain within the normal male range. The physiological consequences of these changes remain to be determined. (*J Clin Endocrinol Metab* 89: 1174–1180, 2004)

IN WOMEN, THE dramatic fall in estrogen production is the biochemical hallmark of the menopause. Whereas no such event occurs in men, the male aging process is associated with a slow, steady decline in gonadal androgen production (1–10). Circulating levels of estrogens also decrease as men age, although this decrease is less dramatic than the decrease in serum androgens (11–13). Thus, testosterone/estradiol ratios are reduced in elderly men, presumably due to increased aromatase activity (14), and there is a high incidence of frank hypogonadism (7, 10).

The clinical significance of the decline in androgens in aging men remains controversial. Unequivocal hypogonadism is associated with a variety of symptoms and physiological alterations that are similar to changes that occur with normal aging in men including decreased libido, fatigue, decreased intellectual ability, decreased lean body mass, increased fat mass, and osteoporosis (15–20). Several studies have explored the role of androgen replacement in elderly men. Whereas most have reported increases in general sense of well-being, libido, muscle size, and lean body mass/body fat ratios, effects on strength and bone density have been more variable (21–26).

The safety of testosterone administration in elderly men, especially with regard to possible effects on the prostate, lipids, and red blood cell production remains a concern despite evidence suggesting that androgen replacement is generally quite safe in healthy aging men (27). Furthermore, although testosterone has been used pharmacologically since the 1940s, available androgen preparations are still not optimal. In particular, oral androgen replacement is limited by both toxicity and lack of efficacy. Anastrozole (Arimidex, AstraZeneca Pharmaceuticals) is a potent and selective orally administered aromatase inhibitor used in the treatment of breast cancer in women (28, 29). Because estradiol is a crucial mediator of hormonal feedback at the pituitary and hypothalamus in men (30–33), aromatase inhibition would be expected to promote pituitary stimulation of testicular testosterone production in men. Thus, anastrozole administration may be a novel means of normalizing testosterone levels in elderly men. To test this hypothesis, and to explore the physiologic effects of short-term aromatase inhibition in el-

Abbreviations: DHT, Dihydrotestosterone; PSA, prostate-specific antigen.

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derly men, we administered anastrozole in a placebo-controlled, double-blind study to elderly men with low levels of circulating testosterone and measured serum androgen, estrogen, and gonadotropin levels. Measures of prostate-related and other safety parameters, libido, and quality of life were also obtained.

Subjects and Methods

Study subjects

Forty-one men between the ages of 62 and 74 yr were recruited by advertisement in accord with institutional guidelines for clinical studies. All subjects were required to have screening serum testosterone levels between 150 and 350 ng/dl and normal serum LH and prolactin levels. Subjects were also required to have normal renal and hepatic function and a hematocrit between 35 and 50%. Subjects were excluded from participation if they had a history of malignancy (except basal cell carcinoma), a prostate nodule on digital rectal examination, a serum prostate-specific antigen (PSA) level greater than 4 ng/ml, a clinical history of acute urinary retention, a history of sleep apnea, significant cardiopulmonary disease, major psychiatric disease, or excessive alcohol or substance abuse. Additionally, subjects were excluded if they were taking any medication known to affect steroid hormone levels or steroid hormone binding protein levels (including androgens, estrogens, glucocorticoids, phenytoin, and carbamazepine).

A total of 126 individuals responded to our recruitment efforts and were screened by an initial blood sampling. Forty-one of these individuals were found to be eligible based on the laboratory criteria listed above. Of these 41, three subjects were found to be ineligible by physical examination and were excluded at that point. One additional subject voluntarily withdrew 3 wk before he completed the protocol due to a decrease in libido and other nonspecific symptoms, leaving 37 men who form the basis of this report. The study was approved by the Human Subject Committee of Partners Healthcare System, and all subjects provided written informed consent.

Randomization and methods

Subjects were randomized by computer-generated assignment to one of three treatment groups. Subjects in group 1 received one 1-mg anastrozole tablet daily for 12 wk. Subjects in group 2 received one 1-mg anastrozole tablet on Monday and Thursday of each week and placebo tablets on the remaining days for 12 wk. Subjects in group 3 received a placebo tablet daily for 12 wk.

Subjects were seen on the General Clinical Research Center at Massachusetts General Hospital every 4 wk for a total of four visits: wk 0 (baseline), wk 4, wk 8, and wk 12. All visits occurred on either a Monday or Thursday, and subjects were instructed to refrain from taking that day's medication until after the visit. Thus, subjects in group 1 would have taken the study drug approximately 24 h before each postbaseline visit (and blood sampling), and subjects in group 2 would have taken the study drug approximately 72–96 h before each postbaseline visit. At the conclusion of each visit, sufficient anastrozole (or placebo) was given to each subject to last until the following visit in weekly dose packs. Compliance was assessed by drug diary and inspection of empty drug containers.

Serum gonadal steroid, gonadotropin, and SHBG levels, routine chemistries, and complete blood count were measured at each visit between 8 and 10 h. Serum PSA was measured at wk 0 and 12. The following questionnaires were also administered at wk 0 and 12: American Urological Association Symptom Index Score (an index of benign prostatic hyperplasia severity) (34), the MOS Short-Form Health Survey (35), and the International Index of Erectile Function (index of male sexual function) (36).

Laboratory measurements

Serum bioavailable testosterone was measured by RIA after ammonium sulfate precipitation as described previously (37, 38). The intra- and interassay coefficients of variation were 8 and 9%, respectively. Serum total testosterone was measured by immunoradiometric assay (Diagnostic Products Corp., Los Angeles, CA). The intra- and interassay coefficients of variation were 10 and 12%, respectively. Serum dihydrotestosterone (DHT) was measured by column chromatography/RIA as described previously (2). The intra- and interassay coefficients of variation were 3 and 8%, respectively. Serum estradiol was measured by RIA (Diagnostic Systems Laboratory, Webster, TX). The intra- and interassay coefficients of variation were 2 and 8%, respectively. Serum estrone was measured by RIA (Diagnostic Systems Lab). The intra- and interassay coefficients of variation were 2 and 6%, respectively. Serum SHBG was measured using an immunometric technique (Immulite, Diagnostic Products Corp.). The intra- and interassay coefficients of variation were 6 and 8%, respectively.

Data analysis

End points were compared between groups by repeated-measures ANOVA with the baseline value included as a covariate. If significant differences were found, pairwise tests were performed to compare individual treatment groups. All P values are two-sided, and $P < 0.05$ was considered statistically significant.

Results

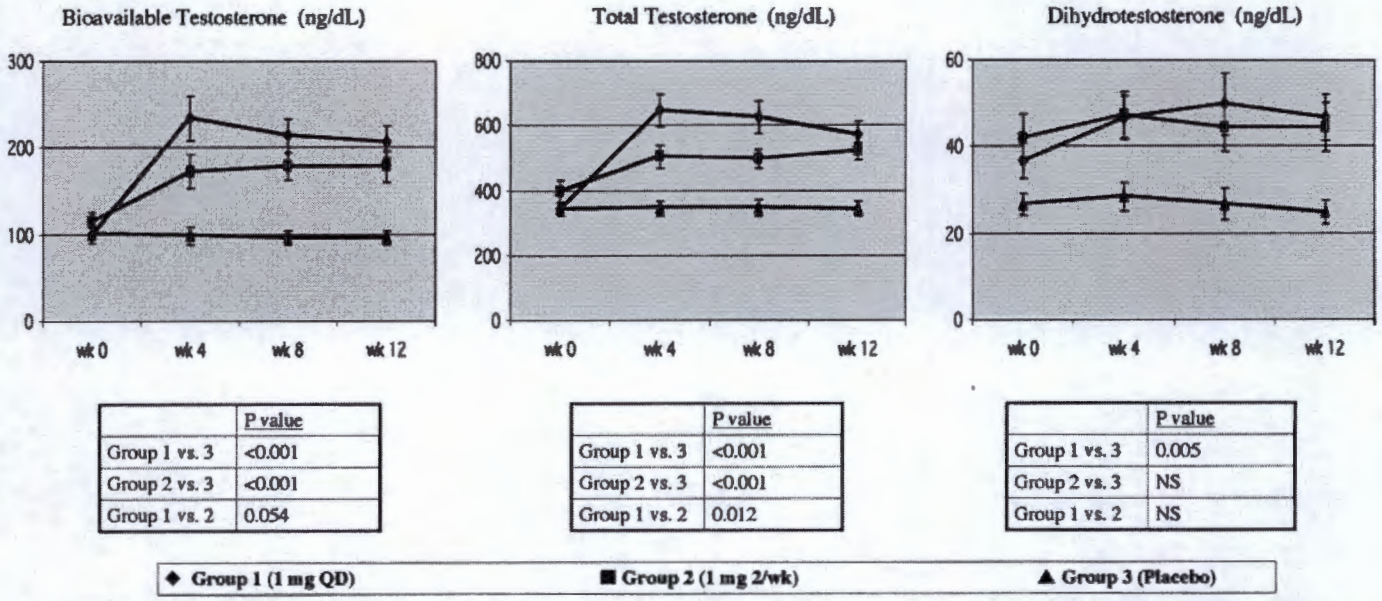
Table 1 shows the baseline clinical characteristics, gonadal steroid levels, and SHBG levels of the study subjects. There were no significant differences among groups.

Figure 1 shows the changes in serum bioavailable testosterone, total testosterone, and DHT during the 12-wk study period. Mean (± SD) serum bioavailable testosterone levels increased from 99 ± 31 to 207 ± 65 ng/dl in group 1 (anastrozole 1 mg daily) and from 115 ± 37 to 178 ± 55 ng/dl in group 2 (anastrozole 1 mg twice weekly) ($P < 0.001$ *vs.* placebo for both groups and $P = 0.054$ group 1 *vs.* group 2). Mean serum total testosterone levels increased from 343 ± 61 to 572 ± 139 ng/dl in group 1 and from 397 ± 106 to 520 ± 91 ng/dl in group 2 ($P < 0.001$ *vs.* placebo for both groups and $P = 0.012$ group 1 *vs.* group 2). Mean serum DHT levels increased from 37 ± 14 to 47 ± 18 ng/dl in group 1 ($P = 0.005$

TABLE 1. Baseline clinical characteristics and gonadal steroid concentrations

	Group 1 (anastrozole, 1 mg QD)	Group 2 (anastrozole, 1 mg twice weekly)	Group 3 (placebo)	Normal range
n	12	11	14	
Age (yr)	67 ± 3	67 ± 3	67 ± 4	
BMI (kg/m^2)	29 ± 5	29 ± 3	28 ± 5	
Bioavailable testosterone (ng/dl)	99 ± 31	115 ± 37	100 ± 34	70–320
Testosterone (ng/dl)	289 ± 37	290 ± 50	274 ± 51	270–1070
Estradiol (pg/ml)	26 ± 8	27 ± 8	23 ± 4	10–50

Values are reported as mean ± SD. No statistically significant differences between groups were observed. To convert values for bioavailable testosterone and testosterone to nmol/liter, multiply by 0.0347. To convert values for estradiol to pmol/liter, multiply by 3.67. BMI, Body mass index.



	P value
Group 1 vs. 3	<0.001
Group 2 vs. 3	<0.001
Group 1 vs. 2	0.054

	P value
Group 1 vs. 3	<0.001
Group 2 vs. 3	<0.001
Group 1 vs. 2	0.012

	P value
Group 1 vs. 3	0.005
Group 2 vs. 3	NS
Group 1 vs. 2	NS

FIG. 1. Mean (±SE) serum androgen levels during the 12-wk study period. To convert values for bioavailable testosterone and testosterone to nanomoles per liter, multiply by 0.0347. To convert DHT to nanomoles per liter, multiply by 0.0344.

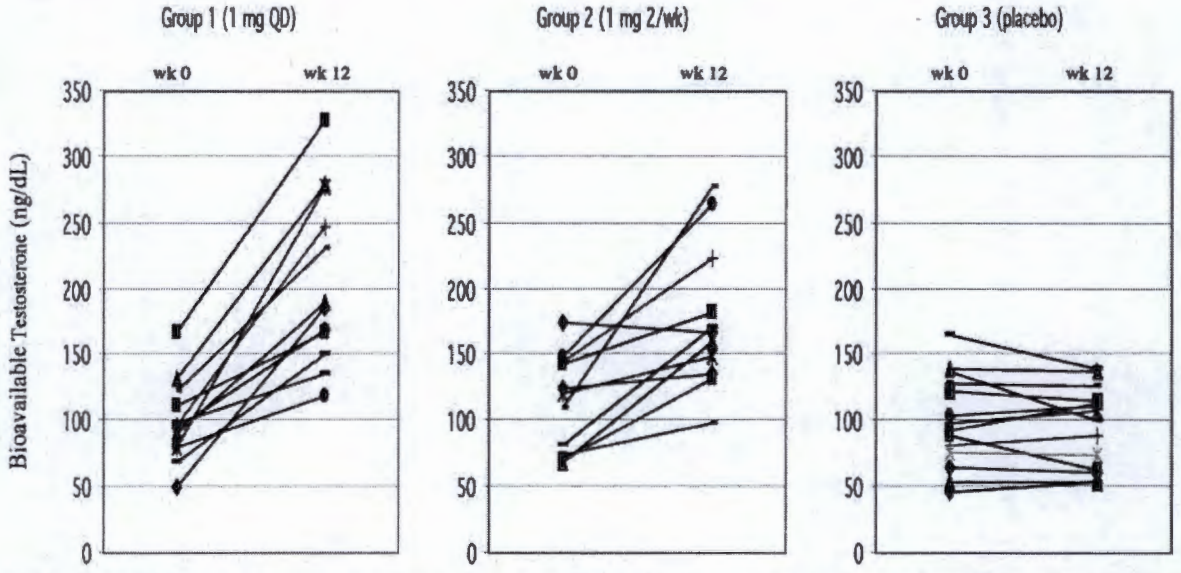


FIG. 2. Individual serum bioavailable testosterone levels at wk 0 and 12. To convert values for bioavailable testosterone to nanomoles per liter, multiply by 0.0347.

vs. placebo), but the increase in group 2 was not statistically different, compared with the placebo group.

Figure 2 shows the individual changes in bioavailable testosterone between wk 0 and 12 in the study subjects divided by group. Levels increased by more than 100% in six of the 12 men in group 1 and in three of the 11 men in group 2 but in none of the men in group 3. In fact, no subject in the placebo group had an increase in bioavailable testosterone of more than 22%.

Figure 3 shows the changes in mean serum estrogens during the 12-wk study period. Mean serum estradiol levels decreased from 26 ± 8 to 17 ± 6 pg/ml in group 1 and from 27 ± 8 to 17 ± 5 pg/ml in group 2 ($P < 0.001$ *vs.* placebo for both groups and P = NS group 1 *vs.* group 2). Notably, the 12-wk serum estradiol levels remained in the normal male range (10–50 pg/ml) in all but one treated subject (group 1 subject, level 9 pg/ml). Mean serum estrone levels decreased from 38 ± 19 to 21 ± 9 pg/ml in group 1 and from 45 ± 16 to 23 ± 7 pg/ml in group 2 ($P < 0.001$ *vs.* placebo for both groups and P = NS group 1 *vs.* group 2). Mean serum SHBG levels decreased from 38 ± 12 to 34 ± 12 nmol/liter in group 1 ($P = 0.015$ *vs.* placebo) but did not decrease significantly in group 2, compared with the placebo group.

Figure 4 shows the change in serum LH levels during the 12-wk study period. Mean serum LH levels increased from 5.1 ± 4.8 to 7.9 ± 6.5 U/liter and from 4.1 ± 1.6 to 7.2 ± 2.8 U/liter in groups 1 and 2, respectively ($P = 0.007$ group 1 *vs.* placebo, $P = 0.003$ group 2 *vs.* placebo, and P = NS group

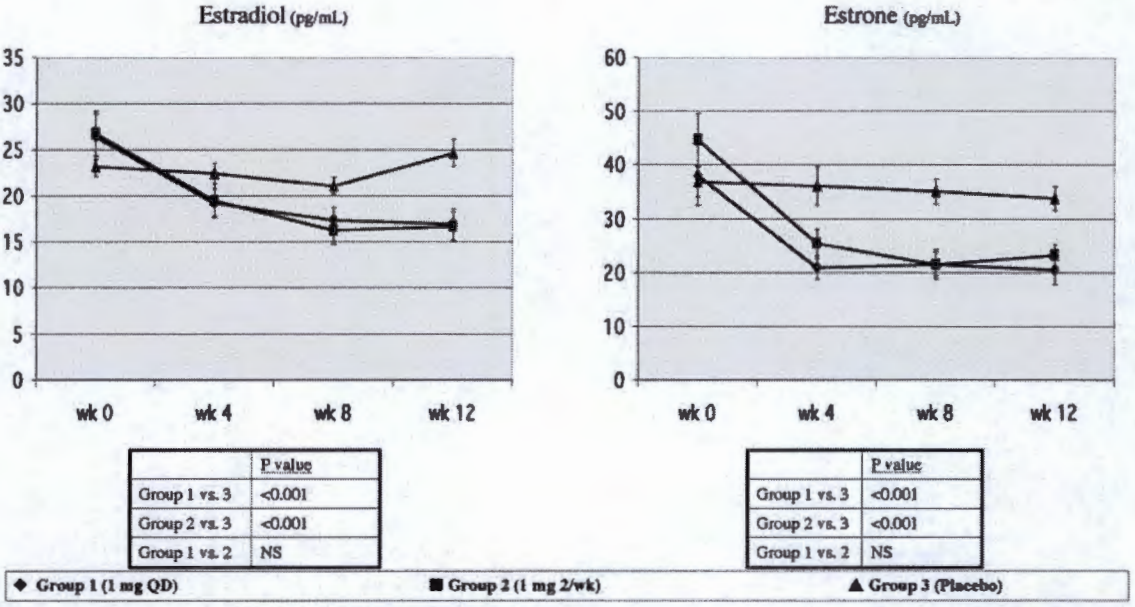


FIG. 3. Mean (±SE) serum estrogen levels during the 12-wk study period. To convert values for estrone and estradiol to picomoles per liter multiply by 37 and 3.67, respectively.

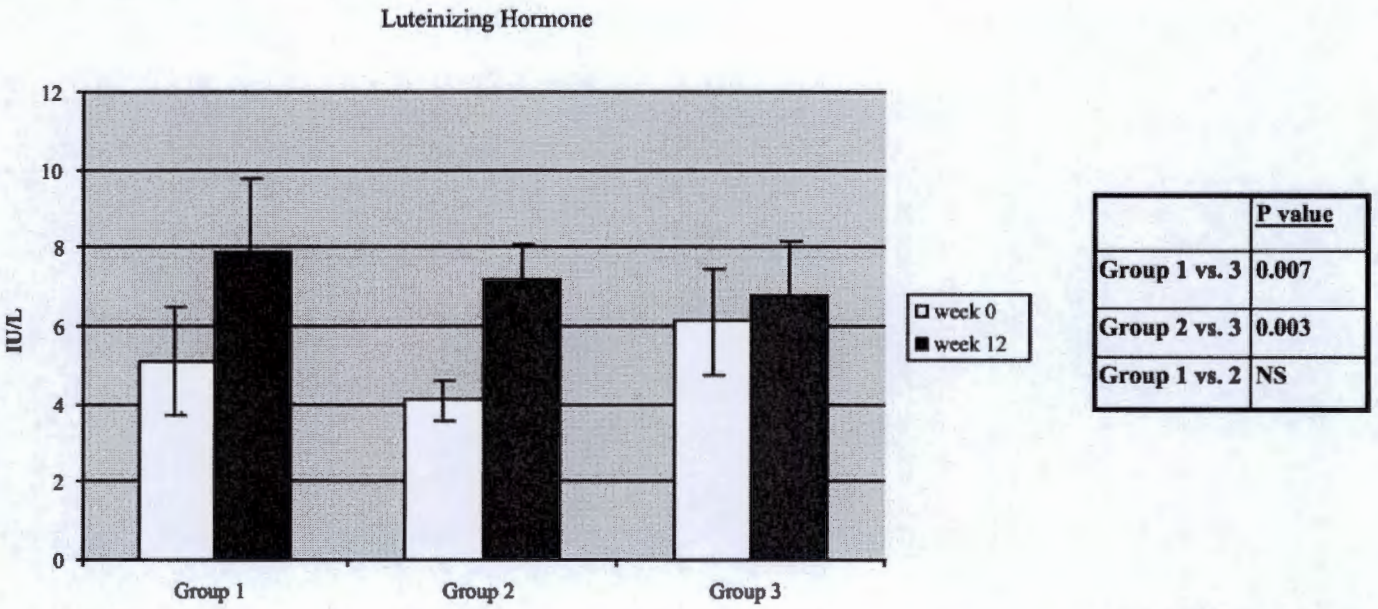


FIG. 4. Mean (±SE) serum LH levels at wk 0 and 12 in men receiving 1 mg of anastrozole daily (group 1), 1 mg of anastrozole twice weekly (group 2), or placebo (group 3).

1 *vs.* group 2). Mean serum FSH levels increased from 10.0 ± 13.9 to 13.9 ± 14.8 U/liter in group 1 and from 7.8 ± 6.5 to 10.3 ± 5.8 U/liter in group 2 ($P < 0.001$ group 1 *vs.* placebo, $P = 0.005$ group 2 *vs.* placebo, and P = NS group 1 *vs.* group 2).

No significant between-group changes were observed in health-related quality of life (MOS Short-Form Health Survey), erectile function (International Index of Erectile Function), or benign prostatic hyperplasia symptoms (American Urological Association Symptom Index Score).

Changes in PSA levels are shown in Fig. 5. Whereas the overall ANOVA was of only borderline significance ($P = 0.076$), PSA levels did increase significantly in group 2 *vs.* the placebo group (1.7 ± 1.0 to 2.2 ± 1.5 ng/ml, $P = 0.031$ group 2 *vs.* controls). PSA did not increase significantly in group 1 *vs.* the placebo group (1.6 ± 0.8 to 1.7 ± 0.8 ng/ml, P = NS group 1 *vs.* placebo). Two patients (both in group 2) had increases in PSA levels from below to above 4 ng/ml during the 12-wk study. Both patients underwent prostate biopsy. In one case, adenocarcinoma was diagnosed and the patient began external beam radiation. In the other case, the biopsy was negative and the subject has since been followed up without incident.

No changes were observed in hematocrit or hemoglobin

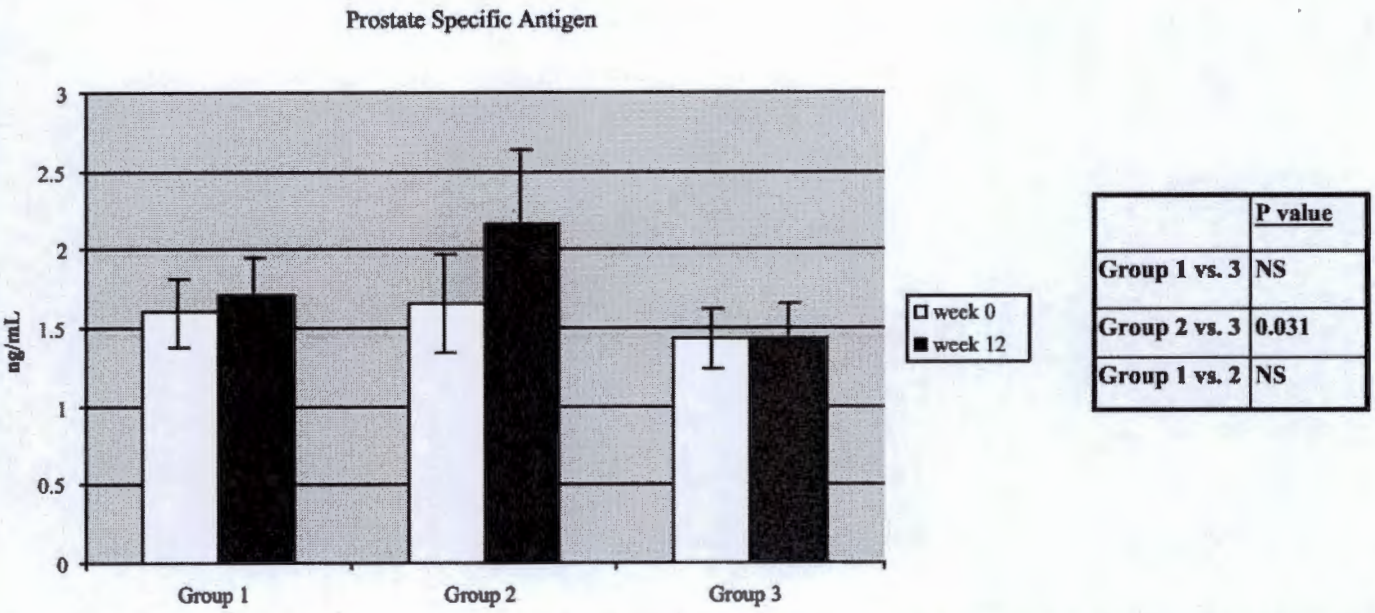


FIG. 5. Mean (±SE) serum PSA levels at wk 0 and 12 in men receiving 1 mg of anastrozole daily (group 1), 1 mg of anastrozole twice weekly (group 2), or placebo (group 3).

levels. Similarly, liver function tests did not increase nor were any other significant toxicities reported.

Discussion

This study demonstrates that aromatase inhibition is an effective means of increasing testosterone production in elderly men with low or borderline low serum testosterone levels. Specifically, in this study we have shown that anastrozole, even at low doses, increases serum LH and testosterone levels robustly while decreasing serum estrogen levels in a more modest fashion.

Current testosterone replacement methods have important limitations. Oral androgens are potentially hepatotoxic and injectable testosterone esters result in supraphysiological peaks of testosterone levels followed by hypogonadal troughs (39). Transdermal testosterone patches frequently cause local skin reactions and are associated with a decline in serum testosterone concentrations toward the end of the treatment period (40). Testosterone gels appear to cause fewer dermatological reactions but can be associated with transmission of testosterone from male patients to female partners (41). Thus, a well-tolerated orally administered agent may have unique potential as a means of androgen replacement therapy. Furthermore, gynecomastia is a common side effect of current modes of androgen administration (27) but does not occur with aromatase inhibition as estrogens are reduced.

Anastrozole's effect on androgen production is likely directly mediated by the reduction in estrogen production. By reducing estradiol synthesis (and hence estradiol's negative feedback on the pituitary and hypothalamus) (30–33), anastrozole appears to stimulate pituitary LH secretion sufficiently to increase endogenous testosterone production to the mid-normal range for young healthy men. Furthermore, this increase in gonadotropin secretion is also likely responsible for limiting the reduction in estrogen levels and maintaining normal (albeit reduced) estradiol levels in treated subjects. In support of this hypothesis, it has previously been shown that GnRH-analog-blocked, but testosterone-replaced, men have much lower serum estradiol levels when given similar doses of anastrozole (42). Thus, the feedback loop of the hypothalamic-pituitary-gonadal axis effectively limits the potency of anastrozole to decrease estradiol production to a degree that may be more physiologically significant.

Whereas it has been well documented that gonadal steroid levels decrease with age in men, the mechanism underlying this decrease has not been defined. In fact, unless testosterone levels are severely depressed or other hormonal abnormalities coexist, the etiology of the hypogonadism is rarely related to overt gonadal or pituitary pathology (43). This observation suggests that more subtle physiological alterations are responsible for the changes in androgen levels observed in aging. For example, it has been suggested that the increase in gonadotropin levels in older men is a result of Leydig cell resistance to pituitary stimulation (2, 5, 8, 10). Alternatively, it has also been hypothesized that the gonadotropin-suppressive activity of androgens are increased in elderly men (44). Finally, as aromatase activity increases with age, an alteration in the estrogen/testosterone ratio may contribute to decreased androgen production (45, 46). Importantly, the effects of aromatase inhibition that we observed in this study are consistent with (but do not prove) a role of increased aromatization in the androgen deficiency in the aging male syndrome.

Anastrozole has been given to men of various ages in several small uncontrolled studies (47–49). In these studies similar changes in gonadal steroid hormone levels were generally observed. Interestingly, in a study of 15 eugonadal elderly men given 2 mg of anastrozole daily for 9 wk, serum

estradiol levels decreased by 29% and total testosterone levels increased by 56% (49). These changes are no greater than those observed in our subjects receiving half the dose and suggest the lack of a continued testosterone dose response beyond the 1-mg level. In fact, serum estradiol levels changed similarly in the two treated groups in the present study, indicating that the optimal dose of anastrozole may depend on the desired end point (decreasing estradiol *vs.* increasing testosterone).

The long-term physiologic effects of the increasing bioavailable testosterone and decreasing estradiol levels in elderly men are unclear. The primary end points in this study were changes in serum testosterone and estradiol concentrations. Whereas quality of life and libido measures were obtained, this study was not sufficiently powered to detect clinically important changes in these end points. Longer-term studies of traditional androgen replacement in men with low or borderline low testosterone levels have shown beneficial effects on these parameters as well as on body composition and bone mineral density (21, 22, 24–26, 50, 51). Because aromatizable androgens were administered in these studies, however, it is possible that similar improvements would not be observed in men receiving anastrozole. This issue may be especially important with regard to bone metabolism, in which the importance of estrogens in maintaining normal bone turnover has been established (42, 52). Additionally, the relative roles of androgens and estrogens in the central nervous system, lipid metabolism, vascular physiology, and cardiovascular risk in general are areas of considerable interest and importance in this population currently, but ones in which no definitive relationship has yet been defined (53).

The safety of androgen replacement in elderly men is currently an area of considerable controversy. Androgen administration in elderly men can cause polycythemia, especially with high doses of parenteral testosterone (22–24). Hematocrit did not increase in the treated subjects in the present study. Prostate size and PSA levels increased in some studies of androgen replacement in elderly men although a causative relationship between testosterone replacement and prostate cancer has not been established (54, 55). In the present study, serum PSA levels increased in subjects receiving the lower anastrozole dose and symptoms related to prostatic obstruction did not worsen in any group. Notably the possibility that estrogens may play a role in the development of both prostate cancer and hyperplasia has recently been suggested from a variety of animal studies (56–59). Specifically, in mice with selective inactivation of the aromatase gene, there was no induction of prostate malignancy despite dramatically elevated androgen secretion from birth (60). Thus, it is conceivable that the prostate-related risk profile of androgen replacement via aromatase inhibition might be different from that for standard androgen replacement therapy.

We conclude that the aromatase inhibitor anastrozole increases androgen production and normalizes serum testosterone levels in older men with mild hypogonadism. Serum estradiol levels are reduced but generally remain within the normal range for men. Longer-term studies are needed to assess the overall physiologic consequences of this combined hormonal alteration in aging men.

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Redox reactions of dehydroepiandrosterone and its metabolites in differentiating 3T3-L1 adipocytes: A liquid chromatographic–mass spectrometric study

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Abstract

Dehydroepiandrosterone is known to depress fatty acid formation in differentiating 3T3-L1 adipocytes. The metabolism of dehydroepiandrosterone and four of its natural metabolites in differentiating adipocytes was studied by liquid chromatography–mass spectrometry. Adipocytes rapidly converted dehydroepiandrosterone to androst-5-ene-3β,17β-diol. 7α-Hydroxy-DHEA was interconverted with 7-oxo-DHEA and 7β-hydroxy-DHEA and the corresponding 17β reduced products. Dehydroepiandrosterone and its derivatives were detected only in the culture medium suggesting that dehydroepiandrosterone is metabolized via enzymes located in close proximity to, or that are integral parts of the cell membrane. Alternatively, there may be efficient mechanisms at play for extrusion of the steroids to the aqueous media rather than being retained in the lipid-rich cell. An interesting aspect of the study was finding androstenediol as the major metabolite of dehydroepiandrosterone. Androst-5-ene-3β,17β-diol has been implicated in prostate cancer. The contribution of adipose cells to the circulating supply of androst-5-ene-3β,17β-diol may therefore be considered in managing prostate cancer.

Keywords: Adipocytes; Steroids; Dehydroepiandrosterone; Androstenediol; Liquid chromatography–mass spectrometry; Metabolism

Dehydroepiandrosterone (DHEA, **I**)[3] and its sulfate ester (DHEA-S) are produced in the adrenal cortex and are the most abundant steroids in human plasma. DHEA reaches its maximum concentration in early adulthood (20–30 years) and decreases steadily to about one-tenth that concentration at age 70–80 [1]. It is a precursor for the formation of androgens and estrogens, and also exerts several physiological effects independent of sex hormones.

DHEA (**I**) increased the size and weight of brown adipose tissue [2] in mice but prevented accumulation of body fat by increasing resting metabolic rate possibly via an increase in futile cycling [3] and heat production [4]. DHEA

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[3] *Abbreviations used:* Adiol, androstenediol or androst-5-ene-3β,17β-diol; Adione, androstenedione or androst-4-ene-3,17-dione; DHEA, dehydroepiandrosterone or 3β-hydroxyandrost-5-en-17-one; 7α-AET, androst-5-ene-3β,7α,17β-triol; 7α-OH-DHEA, 3β,7α-dihydroxyandrost-5-en-17-one; 7β-AET, androst-5-ene-3β,7β,17β-triol; 7β-OH-DHEA, 3β,7β-dihydroxyandrost-5-en-17-one; 7-oxo-DHEA, 3β-hydroxyandrost-5-ene-7,17-dione; 7-oxo-diol,3β,17β-dihydroxyandrost-5-en-7-one; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase/isomerase; CN-IS, 5-pregnen-3β-ol-20-one-16α-carbonitrile used as internal standard for LC–MS; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; FIA, flow injection analysis; LC–MS, liquid chromatography–mass spectrometry; MDI, differentiation cocktail containing methylisobutylxanthine, dexamethasone and insulin.

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(I) depresses lipogenesis [5] and inhibits the differentiation of 3T3 fibroblasts to adipocytes [5]. In differentiating 3T3-L1 preadipocytes, we found DHEA (I) to decrease the formation of Δ^9-16:1 but to increase the formation of Δ^9-18:1 and of stearic acid [6]. The sum of $C_{16} + C_{18}$ fatty acids was not affected.

The mRNAs of three isoforms (type 1, 2 and 3) of 17β-hydroxysteroid dehydrogenase (17β-HSD) are expressed in adipose tissue obtained from the subcutaneous and omental abdominal region of women subjected to elective surgeries. However, only type 3 17β-HSD mRNA seemed to encode for an active protein [7]. Cultured preadipocytes from intra-abdominal adipose tissue of women that had increased type 3 17β-HSD mRNA levels and enzyme activity, converted [8] androst-4-ene-3,17-dione (androstenedione or Adione, **X**) to Testosterone (**IX**). Type 3 17β-HSD is also responsible for the conversion of DHEA (I) to androst-5-ene-3β,17β-diol (androstenediol or Adiol, **II**); type 1 17β-HSD also catalyses this reaction but at a lower rate [9]. The membrane-bound enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) catalyzes an essential step in the transformation of 5-pregnen-3β-ol and 5-androsten-3β-ol steroids into the corresponding Δ^4-3-keto-steroids. 3β-HSD activity is found not only in classic steroidogenic tissues (placenta, adrenal cortex, ovary and testis) but also in several other tissues, including adipose tissue, endometrium, epididymis and skin, thus catalyzing the first step in the intracrine transformation of DHEA (I) into Adione (X) [10].

Because DHEA (I) has a wide variety of weak beneficial effects in experimental animals and humans, efforts have been made to find more active compounds derived from this steroid. In vitro, rat liver rapidly hydroxylates the 7α position of DHEA [11–14] with subsequent conversion to other 7-oxygenated steroids in the sequence DHEA (I) → 7α-hydroxy-DHEA (**III**) → 7-oxo-DHEA (**IV**) → 7β-hydroxy-DHEA (**V**), with branching to diols and triols, and conversion to sulfate esters [14]. The hydroxylation of DHEA (I) at the 7 position [15] to form 7α-OH-DHEA (**III**) was demonstrated in adipose stromal cells [16].

To determine which steroid metabolites are formed in adipose cells, we incubated DHEA (I) and several of its metabolic products with differentiating 3T3-L1 preadipocytes, identified and measured the products using state of the art liquid chromatography–mass spectrometry (LC–MS).

Materials and methods

Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and penicillin–streptomycin mixture were from Gibco-BRL (Gaithersburg, MD). Calf serum was from BioWhittaker (Walkersville, MD), insulin from Eli Lilly (Indianapolis, IN) and methylisobutylxanthine was from Aldrich (Milwaukee, WI). DHEA (I) and dexamethasone were purchased from Steraloids, Newport, RI, USA. Adiol (**II**), 7α-OH-DHEA (**III**), 7-oxo-DHEA (**IV**), 7β-OH-DHEA (**V**), 7-oxo-diol (**VI**, 3β,17β-dihydroxyandrost-5-en-7-one), 7α-AET (**VII**, androst-5-ene-3β,7α,17β-triol), and 7β-AET (**VIII**, androst-5-ene-3β,7β,17β-triol) were synthesized in our laboratory by known procedures [17,18]. The structures of the synthesized compounds were confirmed by nuclear magnetic resonance (^{1}H and ^{13}C) and mass spectrometry; their purity was checked by thin layer chromatography (TLC) and by LC–MS, and was found to be more than 99%. Testosterone (**IX**), Adione (**X**) and 5-pregnen-3β-ol-20-one-16α-carbonitrile (**XI**, CN-IS, the internal standard) were purchased from Sigma. All solvents were of HPLC grade (Aldrich). Solid phase extraction (SPE) cartridges (Oasis-HLB, 3cc) were purchased from Waters Associates.

Instrumentation

The chromatographic system consisted of an Agilent 1100 series LC–MS system, comprised of a capillary pump (G1376A) operated in normal mode, column oven (G1313A), autosampler (G1315A), diode array detector (G1315A), a mass detector (G1946A) and a Gilson fraction collector (FC-203B). Data were acquired and processed using Agilent's LC/MSD Chemstation version A.09.03 software.

Culture and differentiation of 3T3-L1 cells

The 3T3-L1 preadipocyte cell line was cultured and differentiated as described [6]. The cells were cultured in high glucose (HG)-DMEM with 10% calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) in 10-cm plastic petri-dishes until they were 100% confluent. For differentiation, 2-day post-confluent cells (referred to as day 0) were incubated for 48 h in HG-DMEM with 10% FBS, antibiotics and a differentiation cocktail termed MDI, which contains methylisobutylxanthine (115 μg/mL), dexamethasone (390 ng/mL) and insulin (10 μg/mL). After 48 h, the cells were maintained in HG-DMEM with 10% FBS, antibiotics and insulin only. This medium was changed every 2 days until the cells were collected for analysis. Stock solutions of 100 μM DHEA (I), Adiol (**II**), 7α-OH-DHEA (**III**), 7β-OH-DHEA (**V**), 7-oxo-DHEA (**IV**) or 7β-AET (**VIII**), prepared in ethanol, were added at day 0 to a final concentration of 100 μM and replenished with every medium change. Cell culture supernatants were collected on days 8, 10 and 12, and the adipose cells were collected on day 12 and stored at −80°C until assayed. Both the cell supernatants and the cell pellets were analyzed by LC–MS. Non-differentiated (control) 3T3-L1 cells were cultured for 12 days in HG-DMEM with 10% FBS and antibiotics; the supernatant media were changed every 2 days.

Sample preparation for LC–MS analysis of steroids

To 1 mL of culture supernatant, 20 μL of the internal standard (**XI**, CN-IS, 1 μg in 20 μL methanol) was added and mixed. The aqueous layer was extracted with ethyl acetate–hexane (3 mL, 95:5, v/v). The organic layer was separated, evaporated to dryness under nitrogen at 40 °C and the residue, thus obtained, was dissolved in methanol–water (200 μL, 50:50) and 20 μL was subjected to LC–MS analysis on a Zorbax-SB C_{18} analytical column (4.6 × 175 mm, 3.5 μm). The remaining aqueous organic layer was evaporated to ~0.5 mL volume at 40 °C under a slow stream of nitrogen to remove residual ethyl acetate. It was diluted with water (1.5 mL), acidified with acetic acid (20 μL), and applied to a solid phase extraction cartridge containing hydrophilic-lipophilic reversed phase sorbent (Oasis-HLB, 3cc) preconditioned by washing with methanol (2 mL) and water (2 mL). The cartridge was then washed with 5% methanol in water (2× 12 mL) and eluted with methanol (2 mL). The methanol eluent was evaporated at 40 °C under a slow stream of nitrogen, the residue was dissolved in methanol–water (200 μL, 1:1 v/v) and 20 μL was injected on a 3.0 × 1150 mm Zorbax-SB C_{18} column.

The cell pellets (obtained at day 12) were extracted twice with 1 mL methanol, the methanol was evaporated under nitrogen, residue dissolved in methanol–water (200 μL, 1:1) and 20 μL was subjected to LC–MS analysis.

Chromatographic conditions

Chromatography of DHEA (I) and its neutral metabolites was performed on a Zorbax-SB C_{18} analytical column (4.6 × 175 mm, 3.5 μm), protected with a Zorbax SB-C_{18} guard column and maintained at 40 °C.

The flow rate was set at 0.8 mL/min and the eluent was monitored at 205 and 240 nm with a reference wavelength of 360 nm. An acetonitrile–water linear gradient (20–45% acetonitrile in 25 min, 90% acetonitrile at 34 min and back to 20% at 35 min, followed by a 10 min post-run time) was used to analyze solvent extracts. The aqueous extracts were analyzed for sulfate conjugates on a Zorbax-SB C_{18} column (3.0 × 1150 mm) at a flow rate of 0.4 mL/min using a gradient comprised of acetonitrile (containing 3% acetic acid) and 3% aqueous acetic acid. The linear gradient started from 10% acetonitrile to reach 40% acetonitrile in 30 min, and 96% acetonitrile in 38 min; it was brought back to 10% at 40 min. The neutral compounds were analyzed using electro-spray ionization (ESI) in positive mode. Operating conditions, optimized by flow injection analysis (FIA) of DHEA (I), 7-oxo-DHEA (IV) and 7β-AET (VIII), were: drying gas (N_2) 12 L/min; drying gas temperature 350 °C; nebullizer pressure 40 psi; capillary voltage 4500 V; fragmentor voltage 80 V. The samples were run in scan mode. The sulfate conjugates were analyzed in negative ion mode using the technique of 'wrong-way-round' ionization [19–21]. Operating conditions were (FIA): drying gas (N_2) 8.0 L/min; drying gas temperature 350°C; nebullizer pressure 40 psi; capillary voltage 3000 V; fragmentor 80 V.

Calibration and quantitation

The standard stock solutions of steroids (1 mg/mL) were prepared by dissolving separately 10–25 mg of steroid samples in methanol. Working solutions were prepared by serial dilution in the concentration range of 10–1000 ng in 20 μL methanol. Blank medium was used to prepare standard samples in the concentration range 20–1600 ng of each steroid containing 100 ng of the internal standard. The internal standard method was used for the quantitation of DHEA (I) and its metabolites. The peak area ratio of the compound of interest and the internal standard was correlated with the concentration of the steroid, using weighted linear and quadratic curve regression analysis with SPSS 11.5.0 statistical software supplied by SPSS Inc., USA. A separate curve was plotted for DHEA and each of its metabolites.

Results and discussion

DHEA (I) is known to undergo metabolism by several pathways leading to the formation of androgens, estrogens, 7-hydroxylated derivatives and their polar conjugates [14,22,23]. The 7-oxygenated Δ^5-steroids are prone to dehydration [24], the driving force being the formation of resonance-stabilized dienones, dienes and trienes. So precautions were taken to extract the metabolites from the incubation mixture without causing any side reactions. This was done by extracting the incubation medium with ethyl acetate moderated with hexane. The presence of hexane in ethyl acetate helped in layer separation and avoided the partial extraction of polar compounds from aqueous media.

Compared to rat liver tissue, which produces more than 25 different metabolites of DHEA [14], adipocytes seem to be limited in their metabolic pathways. None of the following substances were detected in the present study: estrogens (estradiol, estriol, and estrone), androgens (except for a trace of Testosterone (IX) and Adione (X)), 3,16,17-androstenetriols, oxoandrostenetriols, androstenetetrols, and 16α-OH-DHEA. An interesting observation was the absence of sulfate conjugates, which indicates the lack of either sulfate or sulfotransferase activity in differentiating adipocytes. The DMEM culture medium supplemented with 10% FBS and antibiotics was free of the steroids. Furthermore, neither DHEA (I) nor any of its metabolites were detected in the extracts obtained from the 3T3-L1 adipocyte cell pellets suggesting that DHEA is metabolized via enzymes that are either integral parts of the cell membrane or are located in its close proximity. Alternatively, there may be efficient mechanisms at play for extrusion of the steroids to the aqueous media rather than being retained in the lipid-rich cell. The data supporting an intracellular receptor for DHEA are relatively weak [25] and it is difficult to say whether DHEA acts as a direct receptor ligand. Recent data does support a plasma membrane receptor for DHEA, but this potential receptor is yet to be isolated. DHEA increased nitric oxide release from intact vascular endothelial bovine and human cells, and this action was mediated by a steroid specific, G-protein coupled receptor [26,27].

Fig. 1 is a representative LC–MS extracted ion chromatogram resulting from incubation of 3T3-L1 adipocytes for 48 h in presence (Fig. 1a) and absence (Fig. 1b) of DHEA. The retention times, molecular weights, mass fragmentation patterns, and relative abundances of DHEA and its metabolites are given in Table 1. DHEA (I) and its metabolites were detected and quantified in extracted ion mode based on the most abundant ion. ESI-MS has a relatively narrow linear dynamic range [28] therefore LC–MS data were subjected to linear and quadratic curve fitting regimes. The regression analysis parameters for DHEA (I) and several of its derivatives are given in Table 2.

Conversion of DHEA (I) and Adiol (II) in differentiating 3T3-L1 adipocytes

Metabolism of DHEA (I) was studied in differentiated 3T3-L1 adipocytes at the end of 48 h incubation on days 8, 10 and 12 (Fig. 2). There were no major differences in the metabolic profiling among these days. We also studied metabolite appearances as a function of time in differentiated 3T3-L1 adipocytes in an attempt to understand the sequence of their formation (Fig. 3). Fully differentiated 3T3-L1 adipocytes metabolized DHEA (I) rapidly to Adiol (II). DHEA (I) levels decreased rapidly concomitant with the increase in the levels of Adiol (II), which was detected as early as 10 min after the addition of DHEA (I). The Adiol (II) was the major metabolite, its production was proportional to the incubation period and was maximal (~50%) after 48 h. Other major metabolites observed were 7α-OH-DHEA (III, ~12%) and 7α-AET (VII, ~7%). Adiol (II) regulates systemic resistance against lethal infections in mice [29–31]. It is also a strong activator of androgen receptor, and induces cell proliferation, prostate-specific antigen (PSA) and mRNA expression. Most significantly, hydroxyflutamide (HF) and bicalutamide (BC), commonly used anti-prostate cancer drugs, failed to block Adiol (II)-mediated androgen receptor transactivation [32–35] indicating the presence of alternative sources of Adiol (II) production. Adiol (II) has been found to be present in high concentration in prostate cancer tissue [36]. The contribution of differentiating

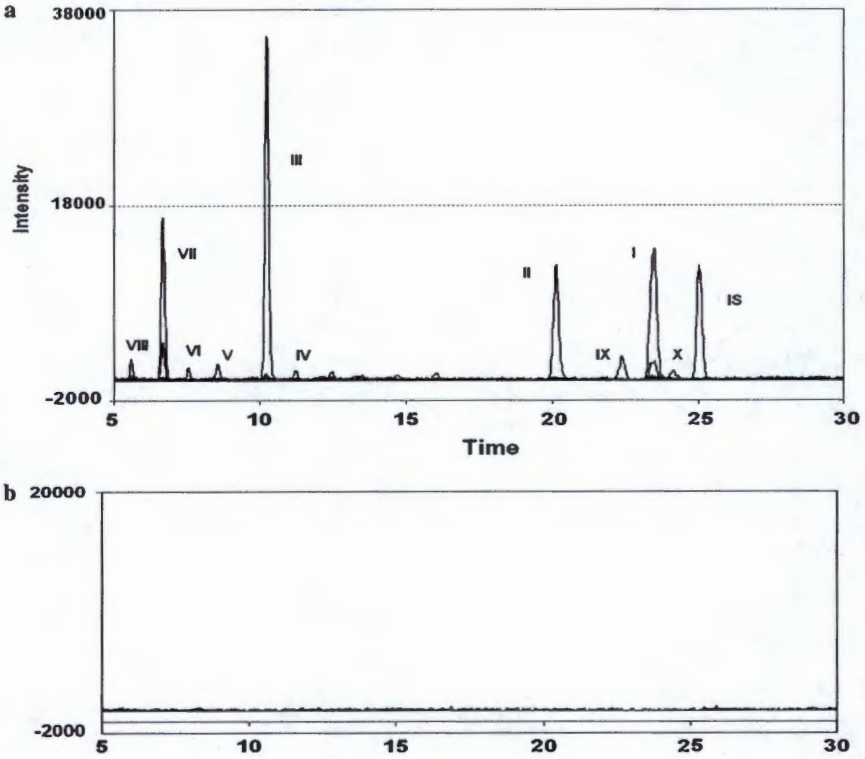


Fig. 1. The extracted ion profile of LC–MS analysis of the supernatant of culture medium of 3T3-L1 adipocytes incubated with 100 μM DHEA for 48 h (a) and without DHEA (b). Cells were differentiated with the MDI cocktail containing 100 μM DHEA as described in Materials and methods. The LC–MS analysis was carried out using a Zorbax SB C_{18} column (4.6 mm × 75 mm, 3.5 μm) eluted with acetonitrile:water gradient ($t = 0$, 20/80; $t = 25$, 45/55 and 90/10 at $t = 34$, v/v) with 0.002% acetic acid at 0.8 mL/min. The sample was analyzed in positive mode at 4500 V with a scan range of *m/z* 200–400. Peak identification: **I**, DHEA (23.5 min); **II**, Adiol (20.1 min); **III**, 7α-OH-DHEA (10.2 min); **IV**, 7-oxo-DHEA (11.2 min); **V**, 7β-OH-DHEA (8.6 min); **VI**, 7-oxo-diol (7.5 min); **VII**, 7α-AET (6.7 min); **VIII**, 7β-AET (5.6 min); **IX**, Testosterone (22.4 min); **X**, Adione (24.1 min); IS, internal standard (pregnen-3β-ol-20-one-16α-carbonitrile, 25.0 min).

Table 1
LC–MS analysis of DHEA and its metabolites produced by incubation with 3T3-L1 adipocytes[a]

Compound[b]	t_R[c] (min)	Characteristic ions in mass spectra; *m/z* (% relative abundance)				
		$(M+Na)^+$	$(M+H)^+$	$(M-H_2O)^+$	$(M-2\times H_2O)^+$	$(M-3\times H_2O)^+$
I	23.5	311 (100)	289 (7)	271 (55)	253 (21)	—
II	20.1	—	—	273 (100)	255 (71)	—
III	10.2	327 (100)	—	287 (23)	269 (65)	251 (6)
IV	11.2	325 (68)	303 (100)	285 (9)	—	—
V	8.6	327 (100)	305 (3)	287 (24)	269 (56)	251 (9)
VI	7.5	327 (93)	305 (100)	287 (1)	—	—
VII	6.7	329 (22)	—	289 (18)	271 (100)	253 (37)
VIII	5.6	329 (35)	—	289 (23)	271(100)	253 (43)
IX	22.4	311 (100)	289 (52)	—	—	—
X	24.1	309 (100)	287 (50)	—	—	—
CN-IS	25.0	364 (100)	342 (5)	324 (5)	—	—

[a] Ethyl acetate–hexane extracts of medium were subjected to LC–MS on a Zorabax-SB C_{18} column. Mass spectra were obtained in the range *m/z* = 200–400.

[b] **I**, DHEA; **II**, Adiol; **III**, 7α-OH-DHEA; **IV**, 7-oxo-DHEA; **V**, 7β-OH-DHEA; **VI**, 7-oxo-diol; **VII**, 7α-AET; **VIII**, 7β-AET; **IX**, Testosterone; **X**, Adione.

[c] Retention time.

adipocytes to the Adiol (**II**) pool may be significant and needs to be further explored in prostate cancer.

Formation of 7α-OH-DHEA (**III**) by differentiating adipocytes indicated the presence of cytochrome P450 7b/P450 1A1/2 [37]. These enzymes have been shown to be involved in the metabolism of DHEA (**I**) and are likely the enzymes responsible for the 7-hydroxylation of DHEA (**I**) in rats. Hydroxylation of DHEA (**I**) at the C7 position has been demonstrated in adipose stromal cells [16], brain microsomal fractions [15], liver [11–14], and in rat prostate [38]. Two different CYPs are said to be responsible for 7α- and 7β-hydroxylation of DHEA in mouse brain microsomes

Table 2
Curve fitting ($X = \alpha Y^2 + \beta Y + \chi$) for DHEA and its metabolites extracted from the cell culture medium[a]

No.	Compound	m/z[b]	α	β	χ	r^{2c}
I	DHEA	271	0.223[d]	—	0.069	0.9990
II	Adiol	273	0.071[d]	—	0.037	0.9992
III	7α-OH-DHEA	269	0.294[d]	—	0.222	0.9953
V	7β-OH-DHEA	269	0.143[d]	—	0.090	0.9997
IV	7-Oxo-DHEA	303	0.892	0.064	−0.090	0.9963
VI	7-Oxo-diol	305	0.659	0.076	−0.070	0.9981
VII	7α-AET	271	0.309[d]	—	0.190	0.9998
VIII	7β-AET	271	0.320[d]	—	0.180	0.9999
IX	Testosterone	289	0.616	0.087	−0.090	0.9945
X	Androstenedione	287	0.958[d]	—	0.050	0.9856

[a] Based on extracted ion chromatogram of mass spectra in positive ion mode.
[b] The extracted ion used for quantitation. CN-IS was used as internal standard.
[c] Correlation coefficient, adjusted.
[d] Weighted linear regression, 1/area^2.

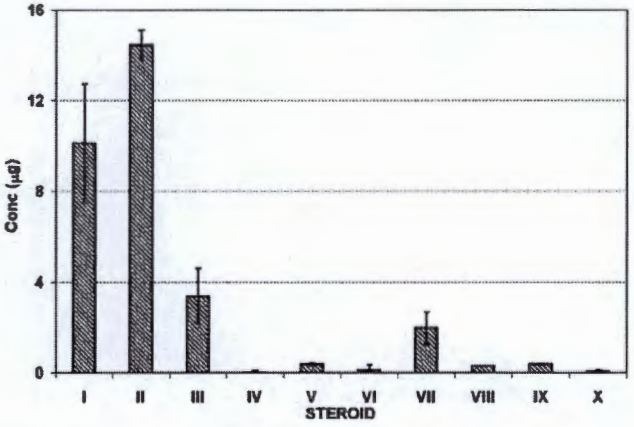


Fig. 2. Conversion of DHEA in differentiating 3T3-L1 adipocytes. Cells were differentiated with the MDI cocktail containing 100 μM DHEA as described in Materials and methods. The medium was replaced every 48 h and DHEA was replenished with every change. Supernatants from day 8, 10 and 12 were collected and stored at −80°C until analysis by LC–MS. Results are mean of LC–MS analysis of 48 h incubation of DHEA with adipocytes on days 8, 10 and 12. **I**, DHEA; **II**, Adiol; **III**, 7α-OH-DHEA; **IV**, 7-oxo-DHEA; **V**, 7β-OH-DHEA; **VI**, 7-oxo-diol; **VII**, 7α-AET; **VIII**, 7β-AET; **IX**, Testosterone; **X**, Adione.

[39]. There is no evidence for direct 7β hydroxylation of DHEA by rat liver tissue [14].

DHEA (**I**) was also metabolized into several other derivatives including Testosterone (**IX**) in small quantities (Figs. 2 and 3). Others were: Adione (**X**), 7β-OH-DHEA (**V**), 7-oxo-DHEA (**IV**), 7β-AET (**VIII**) and 7-oxo-diol (**VI**). Scheme 1 shows the proposed metabolic routes for the formation of each compound: once formed, Adiol (**II**) served as the precursor of Testosterone (**IX**), whereas the production of 7α-OH-DHEA (**III**) preceded that of 7α-AET (**VII**). The concentration of 7α-OH-DHEA (**III**, 3.37 μg) was about nine fold higher than that of 7β-OH-DHEA (**V**, 0.37 μg). We have earlier shown that the DHEA (**I**) was converted to 7α-OH-DHEA (**III**), 7-oxo-DHEA (**IV**), and

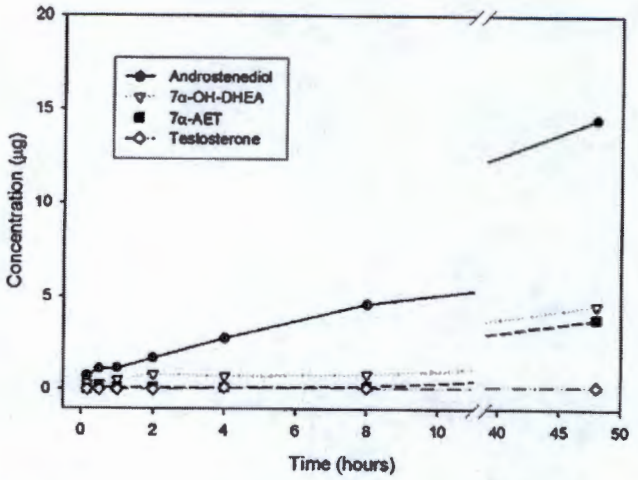
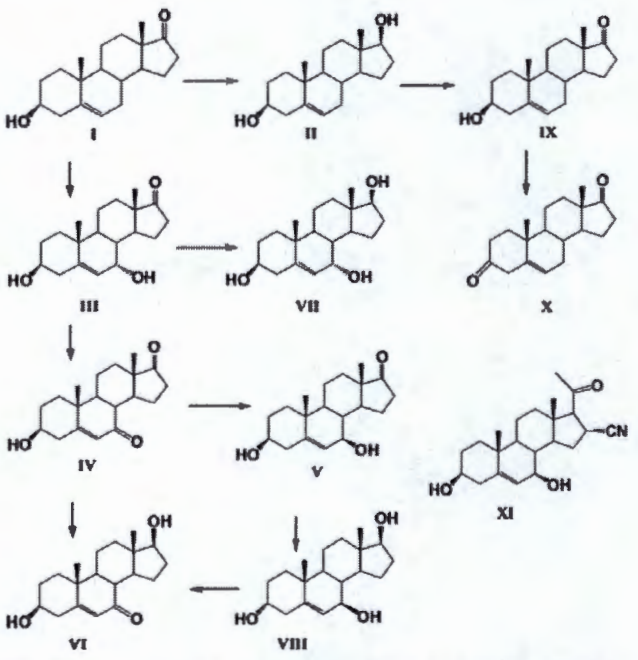


Fig. 3. Metabolism of DHEA (**I**) in fully differentiated 12-day cultures of 3T3-L1 adipocytes. Cells were differentiated with the MDI cocktail as described in Materials and methods. The day of the assay the cells were washed once with warm DMEM medium and incubated with warm DMEM medium supplemented with 10% FBS and antibiotics containing 100 μM of DHEA. Aliquots of the cell culture were taken after 10, 30 min, 1, 2, 4, 8 and 48 h and stored at −80°C until analysis by LC–MS.



Scheme 1. Proposed metabolic route for metabolism of DHEA in differentiating 3T3-L1 adipocytes. **I**, DHEA; **II**, Adiol; **III**, 7α-OH-DHEA; **IV**, 7-oxo-DHEA; **V**, 7β-OH-DHEA; **VI**, 7-oxo-diol; **VII**, 7α-AET; **VIII**, 7β-AET; **IX**, Testosterone; **X**, Adione. **XI**, the internal standard.

7β-OH-DHEA (**V**) in sequence [14]. Formation of negligible amounts of 7β-OH-DHEA (**V**) shows that different enzymes are involved in the redox reactions of 7-oxo-and 17-oxo positions of the steroid molecule. Only a small amount of Testosterone (1.3%) and even smaller amounts of Adione (0.2%) were detected. Adione (**X**) is likely synthesized by differentiating adipocytes from Testosterone (**IX**),

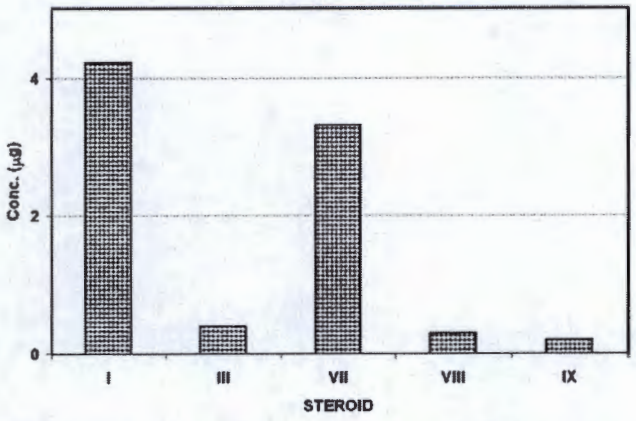


Fig. 4. Conversion of androst-5-ene-3β,17β-diol (Adiol, **II**) in differentiating 3T3-L1 adipocytes. Cells were differentiated with the MDI cocktail containing 100 µM androst-5-ene-3β,17β-diol as described in Materials and methods. **I**, DHEA; **III**, 7α-OH-DHEA; **VII**, 7α-AET; **VIII**, 7β-AET; **IX**, Testosterone.

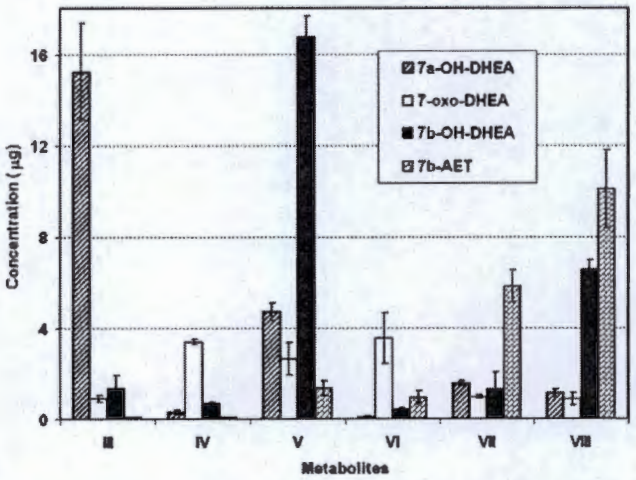


Fig. 5. Conversion of 7α-OH-DHEA, 7-oxo-DHEA, 7β-OH-DHEA and 7β-AET in differentiating 3T3-L1 adipocytes. Cells were differentiated with the MDI cocktail containing 100 µM 7α-OH-DHEA as described in Materials and methods. Results are mean of LC–MS analysis of 48 hr incubation of the steroid with adipocytes on day 8, 10 and 12. **III**, 7α-OH-DHEA; **IV**, 7-oxo-DHEA; **V**, 7β-OH-DHEA; **VI**, 7-oxo-diol; **VII**, 7α-AET; **VIII**, 7β-AET.

since no Adione (**X**) was observed in the incubation of Adiol (Fig. 4). It is well established that Testosterone (**IX**) can be synthesized by two general pathways, depending on the enzymatic composition of individual tissues. The Δ^5 pathway involves pregnenolone → 17-hydroxypregnenolone → dehydroepiandrosterone → Adiol → Testosterone, and the Δ^4 pathway involves pregnenolone → progesterone → 17-hydroxyprogesterone → Adione → Testosterone [40]. Differentiating adipocytes seem to follow a Δ^5 pathway since no Adione (**X**) was detected when Adiol (**II**) was incubated with adipocytes. In contrast to DHEA (**I**), Adiol (**II**) was slowly metabolized and after 48 h only small amounts (<5%) of DHEA (**I**), 7α-OH-DHEA (**III**), 7α-AET (**VII**), 7β-AET (**VIII**) and Testosterone (**IX**) were produced (Fig. 4). Non-differentiated 3T3-L1 preadipocytes cultured in the presence of DHEA (**I**) or any of its derivatives did not metabolize any of them, leaving the parent steroids intact.

Time-course conversion of 7-oxygenated-DHEA derivatives in differentiating 3T3-L1 adipocytes

The 3T3-L1 adipocytes were cultured with either 100 µM 7α-OH-DHEA (**III**), 7β-OH-DHEA (**V**), 7-oxo-DHEA (**IV**) or 7β-AET (**VIII**), and the cell media were collected at days 8, 10 and 12 and analyzed by LC–MS (Fig. 5).

The major metabolite produced from DHEA (**I**) by the 3T3-L1 cells was Adiol (**II**), formed by reduction of the 17-oxo group by 17β-HSD, of which a trace amount was oxidized to Testosterone (**IX**). However the presence of an oxygen function at position 7, made the substrates (**III**–**VIII**) more prone to the redox reactions at allylic position 7 rather than at 17. The major product in the metabolism of 7α-OH-DHEA (**III**, Fig. 5) was 7β-OH-DHEA (**V**), which is produced via the 7-oxo-DHEA (**IV**) intermediate. 7α-AET (**VII**) and 7β-AET (**VIII**) were also formed along with trace amounts of 7-oxo-DHEA (**IV**) and 7-oxo-diol (**VI**). However in the metabolism of 7β-OH-DHEA (Fig. 5) the major product was 7β-AET leading to the conclusion that the 7α-hydroxy steroid is a better substrate for the oxidation at position 7 than a 7β-hydroxy steroid, but the presence of 7β-hydroxy group favors reduction at the 17 position. The equilibrium of the sequence 7α-OH-DHEA (**III**) >7-oxo-DHEA (**IV**) >7β-OH-DHEA (**V**) is shifted towards 7β-OH-DHEA (**V**) in differentiating adipocytes. This is further confirmed by the metabolism of 7-oxo-DHEA (Fig. 5), where the major products were 7-oxo-diol and 7β-OH-DHEA with only small amounts of 7α-OH-DHEA (**III**), 7α-AET (**VII**) and 7β-AET (**VIII**). Differentiating adipocytes metabolized 7β-AET (**VIII**) into 7α-AET (**VII**) understandably via 7-oxidation to 7-oxo-diol (**VI**). 7α-OH-DHEA (**III**) and 7-oxo-DHEA (**IV**) accumulated in trace amounts only (Fig. 5). Finally, since no DHEA (**I**), AED (**II**), or Testosterone (**IX**) were detected when any of the 7-substituted-DHEA derivatives were initially added, modification at position 7 is an irreversible step in DHEA (**I**) metabolism.

The great diversity of biological activities ascribed to dehydroepiandrosterone (DHEA, **I**) probably reflects the conversion and derivatization of the steroid by tissue enzymes. The reports [5,41] that DHEA inhibited the conversion of 3T3 fibroblast clones to adipocytes recognized that the metabolism of DHEA complicated interpretation. The question was examined by Lea-Currie et al. [42] who found that in addition to DHEA, 17β-estradiol, estrone, and pregnenolone inhibited differentiation, whereas DHEAS had no effect.

In differentiating 3T3-L1 cells DHEA (100 µM) increased the levels of stearic and oleic acids (2×) and decreased palmitoleic to less than half of its normal concentration [6]. 7-Oxo-DHEA increased palmitoleic and

palmitic acids slightly but significantly, and had no effect on stearic or oleic acids. 7α-OH DHEA (**III**), 7β-OH DHEA (**V**), and 7β-AET (**VIII**) had no effect [6]. When DHEA was fed to rats (0.5% of the diet) their liver synthesis of fatty acids increased as did the conversion of stearic to oleic acids [43].

In this study we have shown that DHEA is predominantly converted to androst-5-ene-3β,17β-diol (Adiol, **II**), which has been shown to be a strong agonist of the androgen receptor [32]. Because the androgenic activity of Adiol (**II**) is not inhibited by the usual anti-androgenic drugs [32] and because it is concentrated from circulating plasma [36], it appears to play an insidious role in prostate cancer. The contribution of adipose cells to the circulating supply of Adiol (**II**) must therefore be considered in managing prostate cancer.

Acknowledgments

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Biochemistry

Δ^5-Androstenediol is a natural hormone with androgenic activity in human prostate cancer cells

(ARA70/hydroxyflutamide/casodex/testosterone/7-oxo-DHEA)

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Contributed by Henry Lardy, July 29, 1998

ABSTRACT **It is known that androst-5-ene-3β,17β-diol (Adiol), a precursor of testosterone (T), can activate estrogen target genes. The androgenic activity of Adiol itself, however, is poorly understood. Using a transient transfection assay, we here demonstrate in human prostate cancer cells that Adiol can activate androgen receptor (AR) target genes in the presence of AR, and that AR coactivator ARA70 can further enhance this Adiol-induced AR transcriptional activity. In contrast to this finding, an active metabolite of dehydroepiandrosterone, 7-oxo-dehydroepiandrosterone, does not activate AR target gene in the absence or presence of ARA70. Thin layer chromatography analysis reveals that T, dihydrotestosterone, and 17β-estradiol are undetectable in human prostate cancer DU145 cells after treatment with Adiol. Additionally, a proteolysis assay shows that a distinct ligand-receptor conformational difference exists between T-AR and Adiol-AR. Together, the above findings and the fact that T, but not Adiol, can induce transcriptional activity in a mutant AR (mtAR708), suggest that, without being metabolized into T, Adiol itself may represent a natural hormone with androgenic activity in human prostate cancer cells. Because two potent antiandrogens, hydroxyflutamide (Eulexin), and bicalutamide (casodex), that are widely used for the treatment of prostate cancer, fail to block Adiol-mediated induction of AR transcriptional activity in prostate cancer cells, the effectiveness of so-called "total androgen blockage," a standard treatment for prostate cancer, may need to be reevaluated.**

Androst-5-ene-3β,17β-diol (Δ^5-Androstenediol or Adiol), derived from dehydroepiandrosterone (DHEA) and convertible into testosterone (T) (for detail, see Fig. 1) (1), has been suggested to play a role in the regulation of immune responses (2), obesity (3), and the genesis of estrogen-sensitive carcinomas, such as breast cancer (4, 5). Since 1954 (6), Adiol has been known to have estrogenic activity at physiological concentrations (1, 7). Accordingly, the estrogenic effect of Adiol, which is mediated by the estrogen receptor (ER), has been proposed as an essential female hormone that can partially replace the loss of 17β-estradiol (E2) for postmenopausal women. Using transient transfection assay, Kokontis *et al.* (8) also reported that Adiol may convert to 5α-dihydrotestosterone (5α-DHT) and then induce the androgen target gene in human prostate cancer PC-3 cells. The androgenic activity of Adiol, despite its ultimate conversion to T, is poorly understood, and there is no reported evidence of androgen receptor (AR)-mediated effects of Adiol itself. Using the yeast growth assay, the mammalian two-hybrid system, and a transient transfection assay, we investigated the possible androgenic effect of Adiol in the presence of AR and ARA70. Our results suggest that Adiol itself can activate AR transcriptional activity in human prostate cancer cells, and that ARA70 can further enhance Adiol-mediated activation of the AR.

MATERIALS AND METHODS

Chemicals and Plasmids. Adiol, DHEA, androst-5-ene-7,17-dione (Adione), T, DHT, and E2 were purchased from Sigma, 7-oxo-DHEA was synthesized from DHEA as described (9), trilostane was provided by T.-M. Lin (University of Wisconsin, Madison), hydroxyflutamide (HF, Eulexin), and bicalutamide (casodex) were provided by G. Wilding (University of Wisconsin, Madison). pSG5-wild-type AR (wtAR) and pSG5-ARA70 were constructed as described (10), the two mutant ARs, mtAR877 [codon 877 mutation, threonine to serine, derived from a prostate cancer (11)] and mtAR708 [codon 708 mutation, glutamic acid to lysine, derived from a partial androgen insensitive syndrome patient (12)], were provided by S. P. Balk (Beth Israel Hospital, Boston) and H. Shima (Hyogo Medical College, Japan), respectively. pGAL0-mtAR877 and pGAL0-mtAR708 were constructed by inserting fragments—mtAR877 and mtAR708, respectively—into the pGAL0 vector that contained the GAL4 DNA binding domain, as described (13). Similarly, pGAL4-VP16 that contained the GAL4 DNA binding domain-linked to activation domain (AD) of VP16 was used to construct the ARA70 fusion.

Yeast Growth Assay. Yeast cells (gifts from A. J. Caplan, Mount Sinai Medical Center, New York, NY) (14) transformed with the reporter and expression plasmids were grown at 30°C overnight in -3SD medium (-histidine, -leucine, and -tryptophan) with 25 mM 3-aminotriazole (Sigma) and hormones. Yeast transformations were performed by using the modified lithium acetate transformation procedure (15). The cell density was determined from the OD_{660} value.

Cell Culture, Transfections, and Reporter Gene Expression Assays. Human prostate cancer cell lines, DU145, PC-3, and LNCaP, were maintained in DMEM containing 5% fetal calf serum. Transfections and chloramphenicol acetyltransferase (CAT) assays were performed as described (13). Briefly, 4×10^5 cells were plated on 60-mm dishes 24 h before transfection, and the medium was changed to phenol red free DMEM with 5% charcoal-stripped fetal calf serum 1 h before transfection. The cells were transfected by using the calcium phosphate precipitation method. The total amount of DNA was adjusted

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Abbreviations: Adiol or Δ^5-androstenediol, androst-5-ene-3β,17β-diol; DHEA, dehydroepiandrosterone; T, testosterone; ER, estrogen receptor; AR, androgen receptor; E2, 17β-estradiol; Adione, androst-5-ene-7,17-dione; DHT, dihydrotestosterone; HF, hydroxyflutamide; wtAR, wild-type AR; CAT, chloramphenicol acetyltransferase; TLC, thin layer chromatography, MMTV, mouse mammalian tumor virus.

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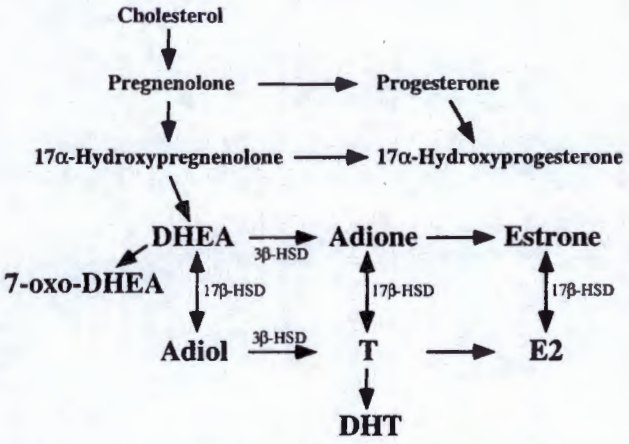

FIG. 1. The steroid biosynthesis pathway involved Adiol and T. 3β-HSD, 3β-hydroxysteroid dehydrogenase.

to 10.5 μg with pSG5 or pVP16 in each transfection assay. Twenty-four hours after transfection, the medium was changed again and the cells were treated with hormones for another 24 h. The cells were then harvested and whole cell extracts were used for CAT assay. Transfection efficiency was normalized by β-galactosidase activity. The CAT activity was quantitated by PhosphorImager (Molecular Dynamics).

Mammalian Two-Hybrid Assay. DU145 cells were transiently cotransfected with a GAL4-hybrid expression plasmid, a VP16-hybrid expression plasmid, and a reporter plasmid pG5CAT. Transfections and CAT assays were performed as described above.

Thin Layer Chromatography (TLC) Analysis. DU145 cells at 4×10^5 cells/dish were cultured for 48 h, and the medium was changed to DMEM with 5% charcoal-stripped fetal calf serum and [^{3}H]Adiol (1 μCi, 23 nM; 1 Ci = 37 GBq) (DuPont/NEN) or [^{3}H]DHEA (1 μCi, 16 nM) (DuPont/NEN). After 24 h, the products were extracted from the medium and cells with diethyl ether and then methanol, as described (16). Aliquots and non-radioactive standard steroids were applied on the same TLC plate. The plate was then exposed twice to toluene: 95% ethanol (9:1). Spots by standard steroids were visualized with specific spray [90% methanol, 5% H_2SO_4, and 5% acetic acid containing p-anisaldehyde (Sigma)], and the running distances were measured. Then, from each lane of the plate 5-mm sections were scraped and radioactivity was determined on the scintillation counter.

Limited Proteolysis Assay. *In vitro* transcriptions/translations were performed in TNT-coupled reticulocyte lysate systems (Promega) in the presence of [^{35}S]methionine. Three-microliter aliquots of labeled translation mixture were incubated for 60 min at room temperature with 3 μl of hormones diluted in water. Then, 1 μl of 25 μg/ml trypsin solution was added, followed by incubation for 15 min at room temperature. The samples were then loaded on 0.1% SDS/12.5% polyacrylamide gel, and autoradiography was carried out as described (13, 17, 18).

RESULTS AND DISCUSSION

Adiol Induces AR Transcriptional Activity. To explore Adiol-mediated AR transcriptional activity, we first investigated the growth of yeast cells containing the human AR and androgen response element (ARE), in the presence of Adiol (Fig. 2A). When treated with 1 nM DHT, the growth of yeast cells that contained wtAR was 3.8× faster than with mock treatment. The presence of 10 nM Adiol stimulated cell growth (2.3-fold increase compared with mock), but 10 nM DHEA did not. We then tested yeast cells that contained mutated AR (mtAR708), which was derived from a partial androgen insensitive syndrome patient, and responds to T and DHT, but not E2, or antiandrogens such as HF (12, 13). When treated with 1 nM DHT, the cells that contained mtAR708 grew as well as those containing wtAR. However, in the presence of Adiol or DHEA, the growth was similar to mock treatment.

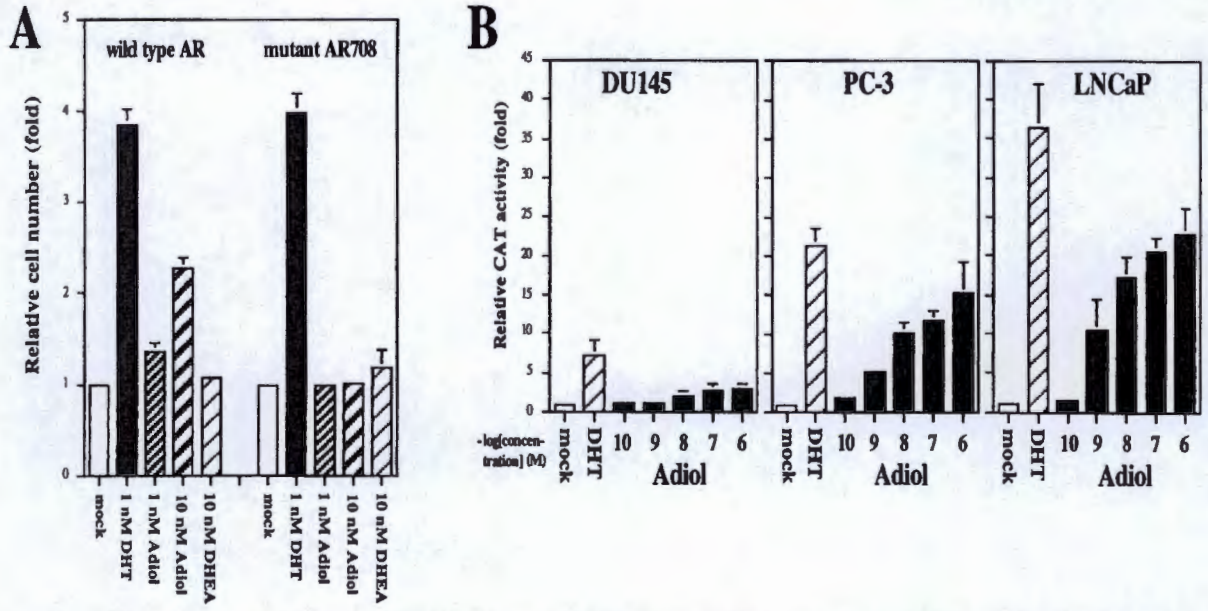

FIG. 2. The effects of Adiol on the growth of yeast cells and on the AR transcriptional activity in human prostate cancer cells. (*A*) Yeast strain, w3031b, transformed with either pG1-wtAR or pG1-mtAR708 and reporter plasmid (pARE-his3) were cultured in -3SD medium (-histidine, -leucine, and -tryptophan) with 25 mM 3-aminotriazole in the absence or presence of hormones as indicated. When the OD_{660} value (X) was between 0.6–1.2, the cell density (Y × 10^7 cells/ml) was calculated by the formula: $Y = 0.276 \times 10^{0.826x}$. Relative cell number represents the means ± SD of three independent experiments. (*B*) DU145, PC-3, or LNCaP cell line was transfected with MMTV-CAT (3.5 μg) and with (for DU145 and PC-3) or without (for LNCaP) wtAR expression plasmid (1.5 μg). Twenty-four hours after transfection, cells were cultured an additional 24 h in the absence or presence of 1 nM DHT or increasing concentrations of Adiol. The CAT activity was determined and the mock treatment was set as 1-fold. Values are the means ± SD of at least three determinations.

The Adiol-induced AR transcriptional activity was further investigated in the prostate cancer cell lines, AR-negative DU145 and PC-3 with cotransfection of an AR plasmid or AR-positive LNCaP carrying endogenous mutated AR (codon 877 mutation, threonine to alanine) (19) without AR plasmid cotransfection. The results were obtained by transient transfection of the ARE-reporter plasmid, mouse mammalian tumor virus (MMTV)-CAT into these cell lines. As shown in Fig. 2*B*, when AR and/or its ARE-reporter were expressed in these 3 cell lines without adding ligands, there was no CAT activity. When treated with 1 nM DHT, CAT activity was induced 7–36-fold over the mock treatment. In the presence of Adiol, CAT activity in DU145 showed only marginal inductions (within 3-fold), whereas the CAT activity in PC-3 or LNCaP was increased up to 15–23-fold in a dose-dependent manner. Interestingly, the induction was detected at the physiological concentration (nM range) of Adiol. Moreover, when MMTV-CAT was replaced with prostate specific antigen-CAT, another AR target gene, similar results were obtained (data not shown). Together, these results indicate that Adiol can activate AR target genes via AR.

ARA70 Enhances Adiol-Induced AR Transcriptional Activity. We have previously reported that ARA70, an AR relatively specific coactivator, can enhance AR transcriptional activity in DU145 cells (10); we thus wanted to know whether ARA70 could enhance Adiol-induced AR transcriptional activity also. As shown in Fig. 3, without cotransfection of ARA70, Adiol can induce the wtAR or mtAR877 transcriptional activity only <3-fold in DU145 cells. However, when ARA70 was cotransfected with wtAR or mtAR877, CAT activities were significantly further induced by Adiol. The level of CAT activity induced was similar to that seen in PC-3 or LNCaP without cotransfection of exogenous ARA70. Similarly, when cotransfected with ARA70 and wtAR or mtAR877, in the presence of DHEA, the inductions can go up to 12-fold in a dose-dependent manner. We also tested the effect of 7-oxo-DHEA, an active metabolite of DHEA that is not metabolically converted to T (9), and could not detect any CAT activity in the presence or absence of ARA70 with AR. These functional differences between two distinct DHEA metabolites (Adiol vs. 7-oxo-DHEA) supplement the previous report that 7-oxo-DHEA is an active metabolite of DHEA (9). When mtAR708 was cotransfected with or without ARA70, at most an only marginal induction was observed in the presence of Adiol, DHEA, or 7-oxo-DHEA. In contrast, when treated with T, transcriptional activity of wtAR or mutants (mtAR877 or mtAR708) was induced 4–8-fold, and ARA70 could enhance the induction by 30–54-fold. This T-mediated induction of mtAR708 transcriptional activity was similar to the effects of DHT-mediated mtAR708 induction as reported (12, 13). The relatively small induction of wtAR or mtAR877 transcriptional activity by DHEA, compared with none by 7-oxo-DHEA, may be due to the metabolic conversion of DHEA to Adiol. (See following section and Fig. 4.)

The Possibility of Conversion of Adiol to T during Transfection. To rule out the possibility that Adiol-induced AR transcriptional activity resulted from the conversion of Adiol to T, four different approaches were applied. (*i*) Trilostane (10 μM; IC_{50} is 1 μM), an inhibitor of 3β-hydroxysteroid dehydrogenase that blocks the conversion of Adiol to T and DHEA to Adione (20, 21), was added in the transfection assay and the results (data not shown) indicated no difference in the presence or absence of this inhibitor. The lack of 3β-hydroxysteroid dehydrogenase in yeast and our demonstration that Adiol-induced AR transcriptional activity in yeast also supports our above finding. (*ii*) TLC analysis (Fig. 4) showed that in a transient transfection assay with or without transfection of AR and ARA70 in DU145 cells, only 7% of [^{3}H]Adiol was

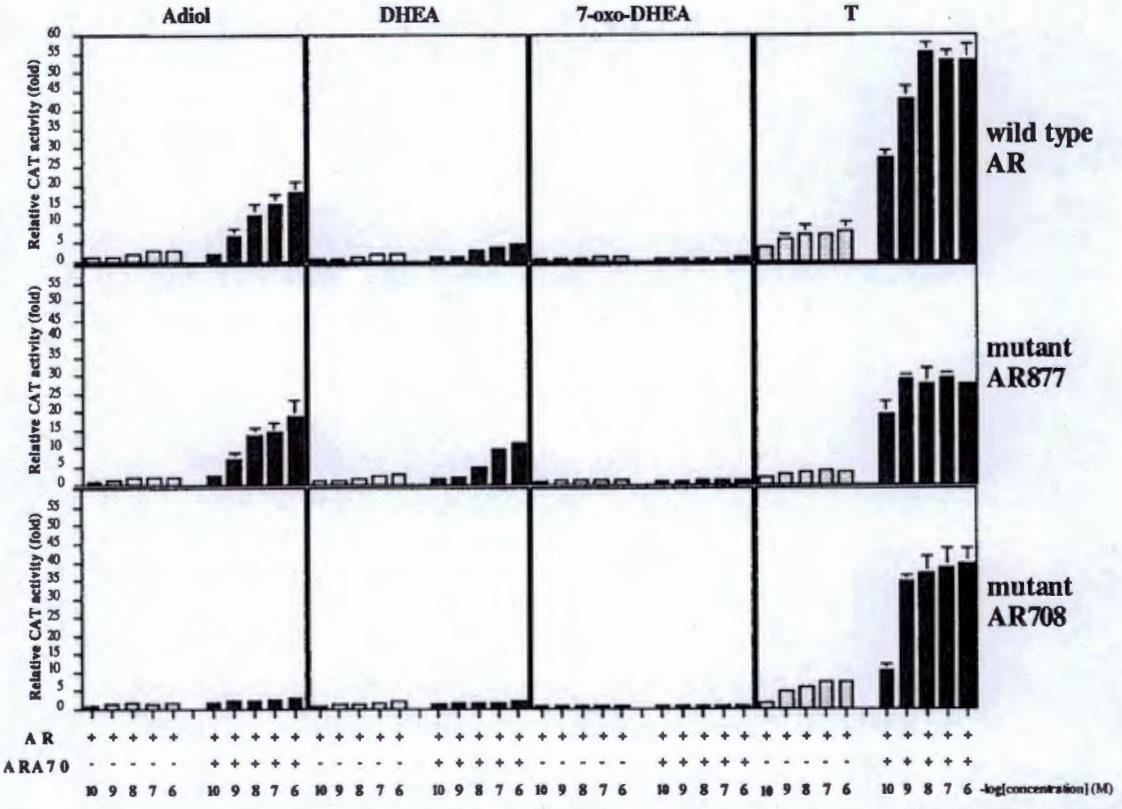

FIG. 3. Activation of AR by Adiol in the presence of an AR coactivator, ARA70, in DU145 cells. DU145 cells first cotransfected with 3.5 μg reporter gene and 1.5 μg AR expression plasmid (wtAR, mtAR877, or mtAR708) with or without 4.5 μg ARA70, were treated with increasing concentrations of Adiol, DHEA, 7-oxo-DHEA, or T. The induction ratios relative to control (mock) are the means ± SD of at least three determinations.

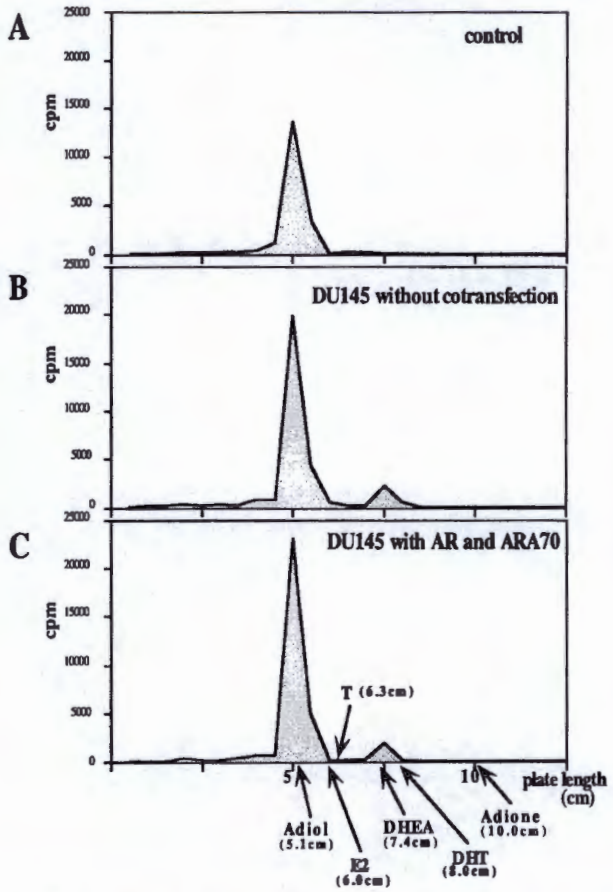

FIG. 4. Metabolite pattern of Adiol in DU145 cells. DU145 cells with (*C*) or without (*B*) cotransfection of AR and ARA70 were incubated with 1 nM [^{3}H]Adiol for 24 h. Steroid metabolites were extracted, dissolved, spotted onto TLC plate, and chromatographed. The plate was then scraped and radioactivity was determined. For the standards, running distances of nonradioactive Adiol, DHEA, Adione, T, DHT, and E2, applied on the same TLC plates, were measured. Control (*A*) was examined without DU145 cells (media only).

converted back to its precursor DHEA. Other Adiol potential metabolites, such as T, E2, DHT, or Adione, were all undetectable. In the same system, 5% of [^{3}H]DHEA could be converted to Adiol, but not other metabolites (data not shown). (*iii*) The results of a limited protease digestion assay (Fig. 5) revealed that, in the presence of T or DHT, only a 29-kDa proteolysis resisting fragment was apparent. In contrast, a specific 35-kDa fragment, as well as the original 29-kDa fragment, was observed in the presence of Adiol. The distinctions between the conformations of the T-AR and Adiol-AR complexes strongly suggest that Adiol-induced AR transactivations are at least structurally different from T-induced AR transactivations. (*iv*) Using wtAR and mtAR708 as comparison, both our yeast growth assay (Fig. 2) and CAT reporter assay (Fig. 3) showed that although T and Adiol can induce wtAR transcriptional activity in the presence of AR and ARA70, only T, but not Adiol, could induce mtAR708 transcriptional activity. This obviously different ability to induce mtAR708 transcriptional activity by T vs. Adiol further strengthens our hypothesis that Adiol has intrinsic androgenic activity, and does not need to be converted to T for this function. With the above four different approaches resulting in similar conclusions, our data therefore strongly support the hypothesis that Adiol-induced AR transcriptional activity may not be due to the conversion of Adiol to T and suggest Adiol itself is a natural hormone with androgenic activity in prostate cancer cells.

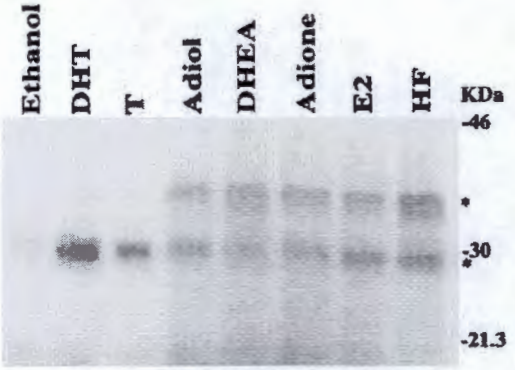

FIG. 5. Conformational changes of the T- or DHT-bound AR vs. Adiol-bound AR. *In vitro* translated AR was incubated with 10 nM DHT, 100 nM T, 10 nM Adiol, 100 nM DHEA, 10 nM Adione, 100 nM E2, 10 μM HF, or 0.01% (vol/vol) ethanol for 60 min at room temperature before limited proteolytic digestion with trypsin. Digestion products were analyzed by electrophoresis on SDS/12.5% polyacrylamide gels and visualized by autoradiography. Trypsin resistant bands are indicated by asterisks. Molecular mass markers are indicated at right.

Adiol-Dependent Interaction between AR and ARA70. To further study the mechanism through which ARA70 enhances Adiol-induced AR transcriptional activity, a mammalian two-hybrid assay was utilized to determine if Adiol can promote the interaction between AR and ARA70. Two fusion plasmids, one of three AR fragments (wtAR, mtAR877, or mtAR708) fused to the GAL4 DNA binding domain, and an ARA70

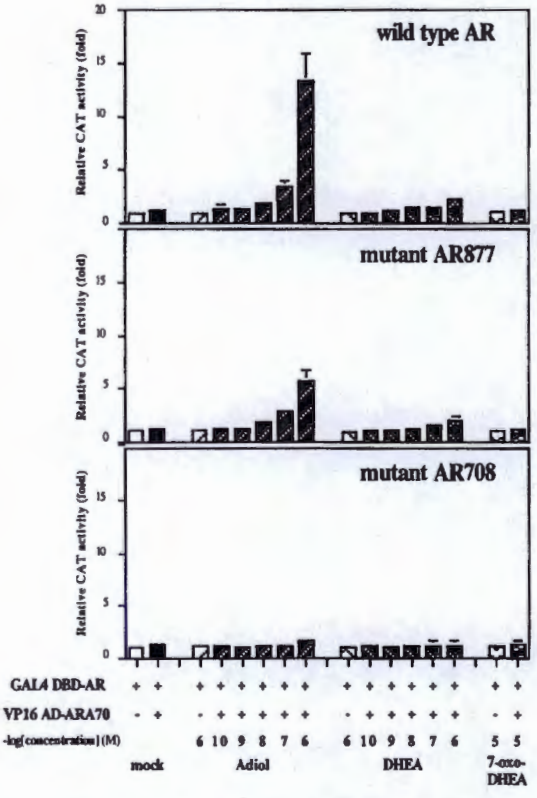

FIG. 6. Dose-dependent physical interaction between AR and ARA70 in the presence of Adiol by mammalian two-hybrid assay. DU145 cells were cotransfected with 3.5 μg pGAL0-wtAR (*A*), 3.5 μg pGAL0-mtAR877 (*B*), or 3.5 μg pGAL0-mtAR708 (*C*), 3.5 μg pVP16-ARA70, and 2.5 μg CAT reporter gene, and treated 24 h with Adiol, DHEA, or 7-oxo-DHEA. Data represent the means ± SD of at least three determinations.

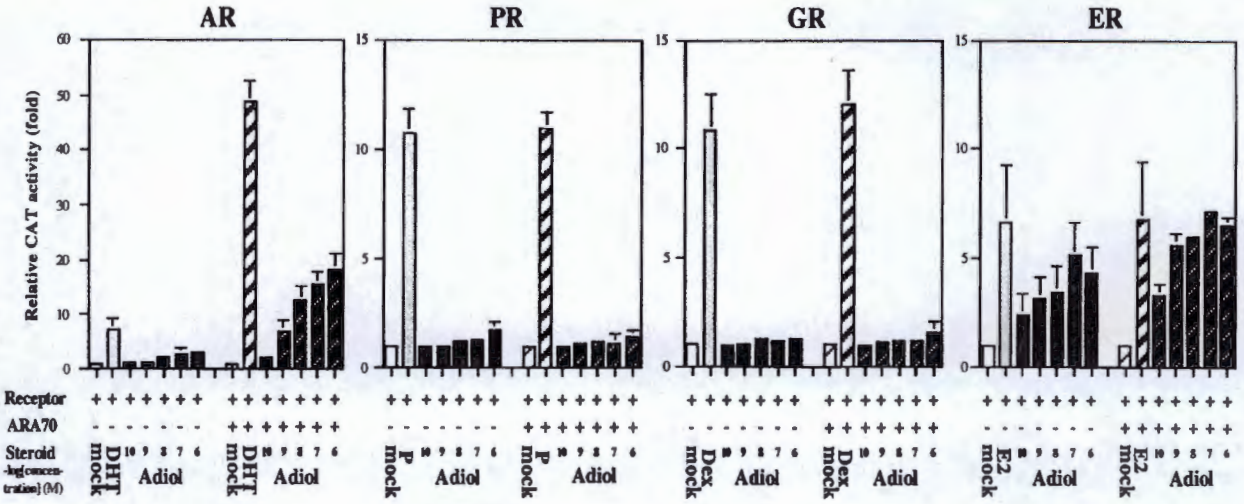

FIG. 7. The effects of Adiol on the transcriptional activity of different steroid receptors. DU145 cells were cotransfected with 1.5 μg receptor and 3.5 μg of its reporter (AR/MMTV-CAT, progesterone receptor (PR)/MMTV-CAT, glucocorticoid receptor (GR)/MMTV-CAT, ER/ERE-CAT) with or without 4.5 μg ARA70, and treated with ligand [1 nM DHT, 10 nM progesterone (P), 10 nM dexamethasone (Dex), or 10 nM E2] or increasing concentrations of Adiol. Values represent the means ± SD of at least three determinations.

fragment fused to the VP16 AD, were coexpressed in DU145 cells with a CAT reporter plasmid. Cells were then treated with Adiol. Thus, the interaction between various AR species and ARA70 in the presence of Adiol could be examined with induced CAT activity. Transient transfection of AR and ARA70 with mock treatment, or transient transfection of either AR or ARA70 with T or Adiol, showed no effect on induction of CAT activity. However, cotransfection of wtAR or mtAR877 with ARA70 increased the CAT activity 3–14-fold in the presence of Adiol (10^{-7}–10^{-6} M) and 2–3-fold in the presence of DHEA (10^{-7}–10^{-6} M) (Fig. 6). In contrast, the addition of a higher concentration (10^{-5} M) of 7-oxo-DHEA showed no activation. As expected, there were no inductions by cotransfection with mtAR708 and ARA70 in the presence of Adiol or DHEA. These results suggest that Adiol itself can promote the interaction between ARA70 and either the wtAR or mtAR877.

The Specificity of Adiol-Induced AR Transcriptional Activity. To evaluate the possibility that Adiol-induced MMTV-CAT activity was mediated by other steroid receptors, such as the progesterone receptor or glucocorticoid receptor, we replaced AR with these receptors in our CAT assay. Because it is known that Adiol can bind directly to the ER and functions as an estrogen (1, 4–7), we also tested the effect of Adiol on ER transcriptional activity using a reporter, ERE-CAT. As shown in Fig. 7, MMTV-CAT activity was induced significantly only via AR, but not progesterone receptor or glucocorticoid receptor, in the presence of ARA70 and Adiol. As expected, ERE-CAT activity could also be induced by Adiol, with the induction fold similar to that achieved by 10^{-8} M E2. These results suggest that Adiol possesses AR-mediated androgenic activity as well as ER-mediated estrogenic activity and can activate the estrogen target gene via ER.

HF and Casodex Fail to Block Adiol-Induced AR Transcriptional Activity. The treatment of advanced stages of prostate cancer, the most commonly diagnosed noncutaneous malignancy in the United States, and the second leading cause of cancer death in North American men, with so-called "total androgen blockage," with a combination of surgical or medical castration with antiandrogen, such as casodex or HF, still has very limited effectiveness (22). Most of the patients will no longer respond to this therapy within 18–24 months of treat-

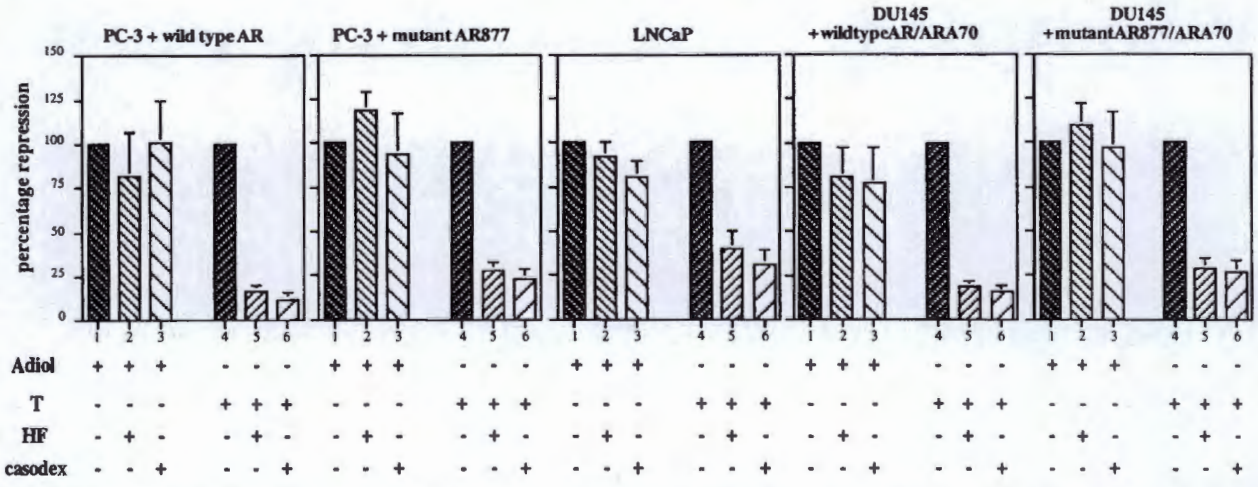

FIG. 8. The effects of antiandrogens on the Adiol-induced transcriptional activity of AR. CAT activity was determined in PC-3 cells transiently cotransfected with wtAR or mtAR877, in DU145 cells with ARA70 and wtAR or mtAR877, or in LNCaP cells without cotransfection of AR expression vector. After transfection, 1 μM HF or 1 μM casodex was added simultaneously with 2 nM Adiol or 1 nM T. The first bars show the activity of Adiol alone or T alone, respectively (set as 100%). Values represent the means ± SD of at least three determinations.

ment. Little or no benefit for the combined approach over castration therapy alone, has been found in patients with regional or metastatic prostate cancer. Labrie *et al.* (23) demonstrated that total androgen blockage caused more than a 90% reduction in serum T level, but only a 26–49% reduction in the serum adrenal androgen level. Blocking the activity of the remaining Adiol (1–2 nM in a patient's serum) may, therefore, be worthy of consideration. To investigate the effects of antiandrogens in conditions mimicking those of patients with advanced prostate cancer, we set up a transient transfection system by using human prostate cancer cell lines. PC-3 and DU145 cotransfected with wtAR or mtAR877, and LNCaP without transfection of the AR, were cultured in media with charcoal-stripped serum, in the presence of 2 nM Adiol plus 1 μM HF or 1 μM casodex. As shown in Fig. 8, these antiandrogens only marginally reduced CAT activities mediated by Adiol, although T-mediated AR transcriptional activity could be reduced significantly. These only marginal reductions were observed irrespective of whether wtAR or mutant AR species were present. This observation suggests that treatment with HF or casodex, in combination with castration may be insufficient to block Adiol's action in AR-positive prostate cancer and may provide a possible explanation for the well documented disappointing clinical findings. This becomes even more of an issue as antiandrogenic therapies, either alone or in combination with castration, have been advocated for earlier stages, and even prevention of prostate cancer (24, 25). The development of new therapeutic approaches that block Adiol's androgenic action, therefore, are worth investigating.

CONCLUSION

In summary, the discovery that Adiol, without conversion to T, has androgenic activity plus the fact that HF and casodex fail to block this Adiol-induced AR transcriptional activity, may not only help us to better understand the molecular mechanisms of Adiol, but may raise critical questions and also open the discussion about the possible role of Adiol in overcoming the effects of androgen ablation therapy for prostate cancer. Because virtually all of the over 39,000 American men who will die of this cancer in 1998 will succumb to disease that is refractory to antiandrogenic therapy, the potential clinical importance of the observations we report here may be considerable.

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ANDROST-5-ENE-7,17-DIONE: A NOVEL CLASS OF SUICIDE SUBSTRATE OF AROMATASE

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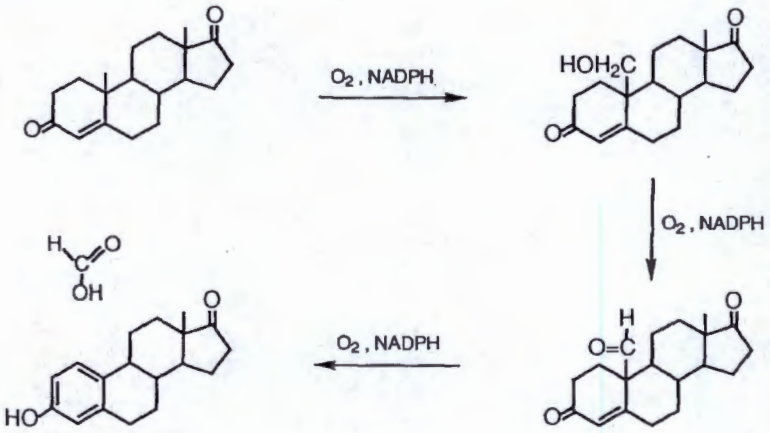
Received May 26, 1992

SUMMARY: 5-En-7-one steroid 1 was found to be a potent inhibitor of aromatase. This along with its 19-hydroxy derivative 7 was characterized as suicide substrate of human placental aromatase (k_{inact}'s of 0.069 and 0.058 min^{-1} and K_I's of 143 nM and 11.1 μM, respectively, for steroids 1 and 7). The results suggest that the 19-oxygenation would be involved in the irreversible inactivation of aromatase by the 5-en-7-one steroids.

Aromatase catalyzes the conversion of 4-en-3-one androgens, androst-4-ene-3,17-dione (androstenedione) and testosterone, to estrogens, estrone and estradiol. The conversions is cytochrome P-450 dependent (1-3) and involves sequential hydroxylations at C-19 as shown in Scheme 1 (4-6). The role of estrogens in promoting some forms of neoplasm, particularly breast cancer, is well documented (7-10). In fact, the estrogen receptor antagonist tamoxifen is used widely in the treatment of breast cancer (11). An alternative approach to blockade of estrogenic activities, which continues to receive attention, involves the lowering of estrogen levels by aromatase inhibition.

A number of potent aromatase inhibitors, analogs of the substrate androstenedione, have been described, including 4-hydroxy (9), 19-ethynyl (12), or 1-methyl-1-ene (13) derivative of androstenedione which is now under clinical evaluation. We have recently developed 3-deoxy C_{19} steroids having a unique 4-ene structure as potent aromatase inhibitors, and demonstrated that a carbonyl function at C-3 of androstenedione is not essential for steroid binding to the active site of aromatase (14-17). As a continuing study of the 3-deoxy steroids as aromatase inhibitors, we have investigated further structure requirements necessary for aromatase inhibition by 5-en-7-one steroids. We report here the synthesis and biochemical evaluation of androst-5-ene-7,17-dione (1) and its 19-hydroxy derivative 7. The 7-one steroids inactivated aromatase in a suicide manner.

0006-291X/92 $4.00



Scheme 1. Proposed mechanism of androstenedione aromatization.

MATERIALS AND METHODS

[1β-^{3}H]Androstenedione (25.4 Ci/mmol) (^{3}H-distribution: β/α=74.2/25.8) was purchased from New England Nuclear (Boston, MA) and NADPH from Kohjin Co. Ltd. (Tokyo, Japan). Human placental microsomes (particles sedimenting at 105,000 x g, washed with 0.5 mM dithiothreitol) were obtained essentially as described by Ryan (18). Androst-5-ene-7,17-dione (1) was synthesized according to the method (19) previously reported.

3β-Acetoxy-19-(tert-butyldimethylsiloxy)androst-5-ene-7,17-dione (3). To a stirred mixture of 3β-acetoxy-19-(tert-butyldimethylsiloxy)androst-5-en-17-one (2) (1.37 g, 2.97 mmol) in benzene (50 ml) and Celite (4.82 g) was added pyridinium dichromate (6.62 g, 17.60 mmol) followed by the addition of 70% tert-butyl hydroperoxide (4.4 g, 48.12 mmol) at 10°C (20). After 15 min at 10°C, the reaction mixture was stirred at room temperature for 24 h under dark. AcOEt (200 ml) was added, and the mixture was filtered through a pad of Celite and washed with 50 ml portions of AcOEt. Combined filtrate was washed with 10% $Na_2S_2O_3$ solution and saturated NaCl solution and dried (Na_2SO_4). After evaporation of the solvent, the residue was recrystallized from acetone-hexane to give 3 (1.04 g, 74%) as colorless plates: mp 145-146°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.04 and 0.07 (3H each, s, 19-OSiMe_2), 0.86 (9H, s, 19-OSi(Me_2)CMe_3), 0.92 (3H, s, 18-Me), 2.06 (3H, s, 3-OCOMe), 3.85 (1H, d, J=10.7 Hz, 19-Ha), 3.94 (1H, d, J=10.7 Hz, 19-Hb), 4.75 (1H, m, 3α-H), 5.93 (1H, d, J=1.5 Hz, 6-H). IR (KBr): 1730, 1700, and 1650 (C=O) cm^{-1}. UV λ_{max} (95% EtOH): 234 nm (ε=1.40 x 10^4). Anal. Calcd for $C_{27}H_{42}O_5Si$: C, 68.31; H, 8.92. Found: C, 68.37; H, 8.86.

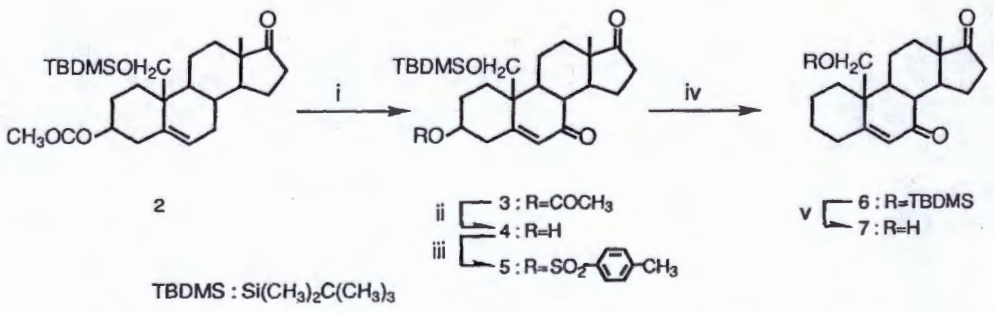
3β-Hydroxy-19-(tert-butyldimethylsiloxy)androst-5-ene-7,17-dione (4). To a solution of compound 3 (1.0 g, 2.11 mmol) in MeOH (50 ml) was added 1 M NaOH solution (5.87 ml). The mixture was stirred at room temperature for 1 h and then poured into saturated NaCl solution (500 ml). The precipitates were collected by filtration, washed with water, dried under vacuum, and recrystallized from acetone-hexane to afford 4 (0.89 g, 98%) as colorless needles: mp 156.5-157°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.03 and 0.06 (3H each, s, 19-OSiMe_2), 0.85 (9H, s, 19-OSi(Me_2)CMe_3), 0.92 (3H, s, 18-Me), 3.72 (1H, m, 3α-H), 3.84 and 3.94 (1H each, d, J=10.7 Hz, 19-H_2), 5.92 (1H, d, J=1.5 Hz, 6-H). IR (KBr): 3350 (OH), 1730 and 1660 (C=O) cm^{-1}. UV λ_{max} (95% EtOH): 239 nm (ε=1.34 x 10^4). Anal. Calcd for $C_{25}H_{40}O_4Si$: C, 69.40; H, 9.32. Found: C, 69.03; H, 9.37.

3β-(p-Toluenesulfonyloxy)-19-(tert-butyldimethylsiloxy)androst-5-ene-7,17-dione (5). p-Toluenesulfonyl chloride (2.0 g, 10.5 mmol) was added to a stirred solution of compound 4 (0.89 g, 2.06 mmol) in pyridine (12 ml) at 0°C. The reaction mixture was stirred at room temperature for 24 h and then poured into chilled water (200 ml). The precipitates were collected by filtration, dried under vacuum, and recrystallized from MeOH to give 5 (1.1 g, 91%) as colorless needles: mp 110-111°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.01 and 0.04 (3H each, s, 19-OSi$\underline{Me}_2$), 0.83 (9H, s, 19-OSi(Me_2)C$\underline{Me}_3$), 0.89 (3H, s, 18-Me), 3.80 and 3.87 (1H each, d, J=10.8 Hz, 19-H_2), 4.38 (1H, m, 3α-H), 5.82 (1H, s, 6-H), 7.36 and 7.82 (2H each, d, J=7.8 Hz, aromatic protons). IR (KBr): 1732 and 1662 (C=O) cm^{-1}. Anal. Calcd for $C_{32}H_{46}O_6SSi$: C, 65.49; H, 7.90. Found: C, 65.00; H, 7.64.

19-(tert-Butyldimethylsiloxy)androst-5-ene-7,17-dione (6). Zinc powder (0.86 g, 13.15 mmol), NaI (1.0 g, 6.67 mmol), and water (0.86 ml) was added to a solution of compound 5 (1.0 g, 1.70 mmol) and the reaction mixture was heated under reflux for 4 h (21). After this time, the mixture was diluted with AcOEt, washed with saturated NaCl solution, and dried (Na_2SO_4). Evaporation of the solvent yielded an oil which was purified by silica gel column chromatography (hexane/AcOEt) followed by recrystallization from MeOH gave 6 (378 mg, 53%) as colorless needles: mp 114-115°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.02 and 0.05 (3H each, s, 19-OSi$\underline{Me}_2$), 0.85 (9H, s, 19-OSi(Me_2)-C$\underline{Me}_3$), 0.92 (1H, s, 18-Me), 3.83 and 3.79 (1H each, d, J=10.7 Hz, 19-H_2), 5.87 (1H, s, 6-H). IR (KBr): 1737 and 1666 (C=O) cm^{-1}. UV λ_{max} (95% EtOH): 240 nm (ε=1.20 x 10^4). Anal. Calcd for $C_{25}H_{40}O_3Si$: C,71.24; H, 9.96. Found: C, 71.16; H, 9.61.

19-Hydroxyandrost-5-ene-7,17-dione (7). To a solution of compound 6 (134 mg, 0.32 mmol) in THF (1.5 ml) was added tetra-n-butylammonium fluoride (245 mg, 0.96 mmol). The reaction mixture was allowed to stand at room temperature for 16 h, poured into water (100 ml), and extracted with AcOEt (100 ml). The organic layer was washed with 5% $NaHCO_3$ solution and saturated NaCl solution, dried (Na_2SO_4), and evaporated to give a solid. Recrystallization of the product from acetone yielded 7 (85 mg, 86%) as colorless prisms: mp 190-190.5°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.94 (1H, s, 18-Me), 3.88 (1H, d, J=11.2 Hz, 19-Ha), 4.09 (1H, d, J=11.2 Hz, 19-Hb), 5.94 (1H, d, J=1.0 Hz, 6-H). IR (KBr): 3350 (OH), 1740 and 1660 (C=O) cm^{-1}. UV λ_{max} (95% EtOH): 242 nm (ε=1.17 x 10^4). Anal. Calcd for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.49; H, 8.54.

Studies of Competitive Inhibition and Time-Dependent Inactivation of Aromatase in Human Placental Microsomes. Aromatase activity was measured by release of tritiated water from [1β-^{3}H]androstenedione during aromatization



Reagents : (i) pyridinium dichromate , tert-BuOH , Celite , benzene ; (ii) NaOH , MeOH
(iii) p-TsCl , pyridine ; (iv) NaI , Zn , $(CH_2OCH_3)_2$; (v) $(n\text{-}Bu)_4NF$, THF

Scheme 2. Synthesis of compound 7.

principally as described by Thompson and Siiteri (1). The inhibition and inactivation studies with compounds 1 and 7 were carried out essentially according to the previous method (16) (Scheme 2).

RESULTS AND DISCUSSION

5-En-7-one steroid 1 was a potent inhibitor of aromatase in human placental microsomes while its 19-hydroxy derivative 7 was a moderately good one (Table 1). Lineweaver-Burk and Dixon plots were linear and showed that the inhibition was competitive (Figure 1). In each set of competition experiments, an apparent K_m (59 ± 5 nM) for androstenedione as well as an apparent K_i for each steroid (K_i: 300 nM or 15 μM for 1 or 7) was obtained. Compound 1, which is a geometrical isomer of the substrate androstenedione, was a potent competitive inhibitor of aromatase while an introduction of a hydroxy group at C-19 of it provides lowered affinity for the active site. The 19-hydroxy derivative of androstenedione, an intermediate of estrone biosynthesis, has a lower binding affinity for the active site than the parent steroid (2,3). This along with the present results suggests that the 7-one steroids may approach the active site in a similar way to the natural substrate and intermediate, in which hydrogen bonding through the carbonyl oxygen at C-7, instead of that through the C-3 carbony oxygen in a series of androstenedione (22,23), may be involved in their bindings to aromatase.

Two steroids were then tested for their abilities to cause a time-dependent irreversible inactivation of aromatase. Time-dependent, pseudo-first order inactivation was observed when both compounds were separately incubated in the presence of NADPH in air (Figure 2). With increasing inhibitor concentrations, increasing apparent rate constants for inactivation (k_{obsd}'s) were obtained for both compounds. Double-reciprocal plots of k_{obsd} versus inhibitor concentration (24) was linear and gave the apparent K_I's of 143 nM and 11.1 μM and overall rate constants for inactivation (k_{inact}'s) of 0.069 and 0.058 min^{-1}, respectively, for compounds 1 and 7 (Figure 2B). The similarities of the apparent K_I's with the apparent K_i's

Table 1. In vitro aromatase inhibitory activity

Compound	IC_{50}, μM[a]	K_i, nM	Inhibition
1	8.1	300	competitive
7	135	15000	competitive
androstenedione	1.0	59 (K_m)	

[a] Substrate: 1 μM [1β-^{3}H]androstenedione

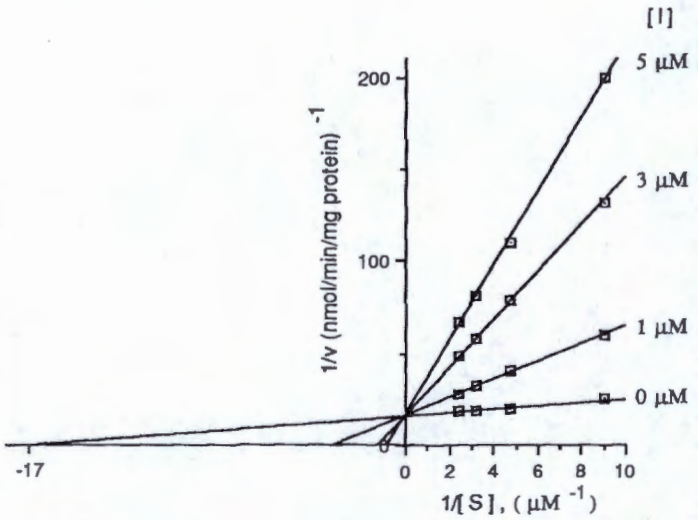


Figure 1. Lineweaver-Burk plot of inhibition of human placental aromatase by compound 1 with androstenedione as a substrate. Each point represents the mean of duplicate determinations. The inhibition experiment with compound 7 gave an essentially similar plot to Figure 1, showing that this is also a competitive inhibitor of aromatase (data not shown).

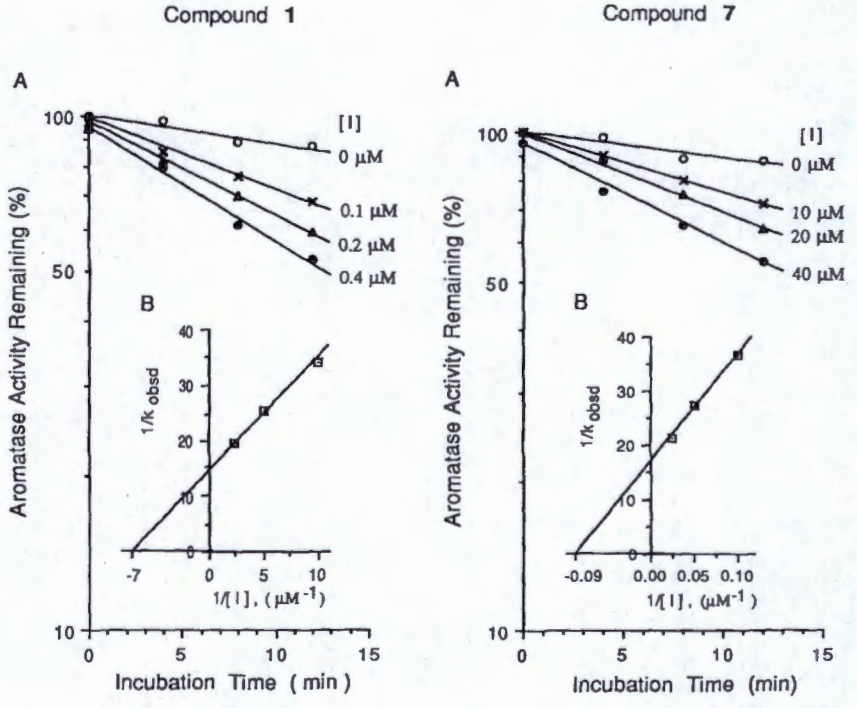


Figure 2. Time- and concentration-dependent inactivation of human placental aromatase by compound 1 or 7 in the presence of NADPH in air (A), and double-reciprocal plot analysis of the kinetics data from A (B).

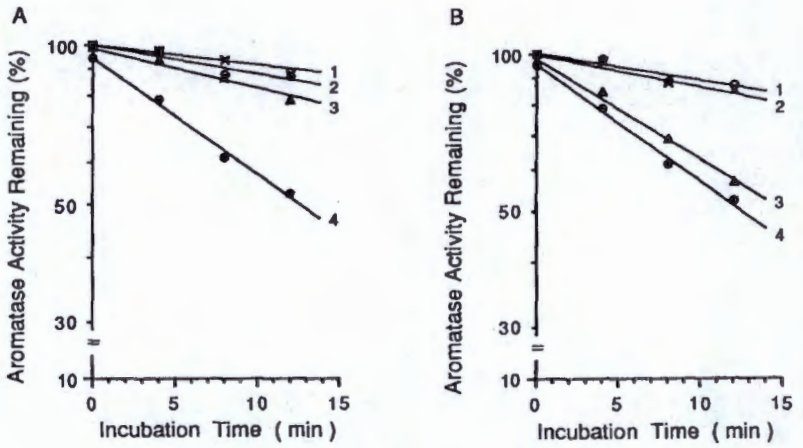


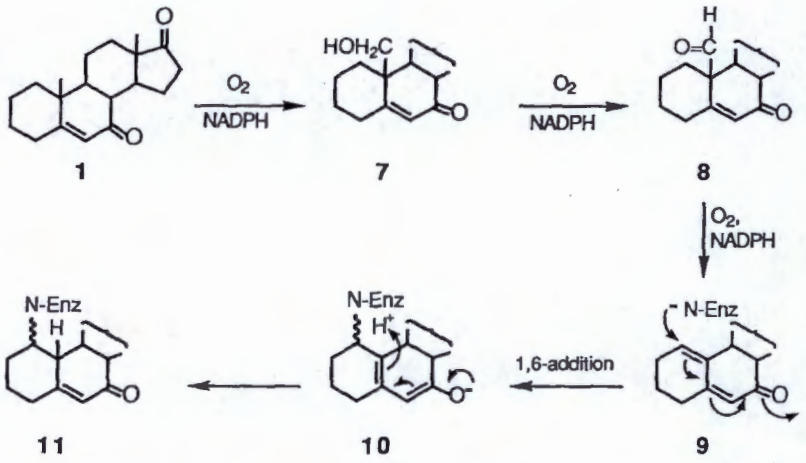
Figure 3. Inactivation of human placental aromatase by compound 1 under various conditions: (A) In the absence of NADPH (line 1) or in nitrogen atmosphere (line 3), the inhibitor (0.4 μM) failed to produce inactivation. Androstenedione at 0 or 1 μM (line 4 or 3) was incubated with aromatase, the inhibitor (0.4 μM), and NADPH in air and protected the enzyme from the inactivation. Control sample (line 2) contained no inhibitor. (B) In the presence (line 3) or absence (line 4) of L-cysteine (0.5 mM), a pseudo-first-order inactivation by the inhibitor (0.4 μM) was observed. Control sample with (line 2) and without (line 1) L-cysteine contained no inhibitor. The inactivation experiments with compound 7 in the absence of NADPH, in nitrogen atmosphere, and in the presence of L-cysteine or androstenedione gave essentially similar results to Figure 3 (data not shown).

obtained from the competitive experiments (Table 1) suggest the initial binding of the inhibitors to aromatase is rate-limiting.

NADPH and oxygen were essential for the activity loss and the substrate androstenedione completely blocked the inactivation (Figure 3A), while a nucleophile, L-cysteine, failed to protect aromatase from the inactivation (Figure 3B). Thus, covalent-bond formation between the enzyme and the reactive intermediate appears to occur rapidly at the active site, therefore, preventing diffusion of the activated inhibitor, a reactive electrophile, in the surrounding media.

The mechanism of aromatase inactivation by compound 1 is presumed to be as follows (Scheme 3); aromatase attacks the 19-carbon of steroid 1 to produce the 19-oxo derivative 8 through 19-alcohol 7, and further oxygenation of compound 8 involves the cleavage of C_{10}-C_{19} bond to generate a reactive electrophile, 1(10),5-dien-7-one 9, which may be attacked by an active site nucleophile in a 1,6-addition manner leading to covalent modification of the enzyme.

The present findings are the first that steroids having a 5-en-7-one system inactivate aromatase in a suicide (mechanism-based) manner, and may have practical applications in drug design of specific inhibitors. At a more fundamental level, presumably the steroids could be play an important



Scheme 3. Proposed mechanism of time-dependent inactivation of aromatase by 5-en-7-one steroids 1 and 7.

role in a understanding of the aromatization mechanism of which details are still a subject of debate.

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ELSEVIER

Studies directed toward a mechanistic evaluation of aromatase inhibition by androst-5-ene-7,17-dione

Time-dependent inactivation by the 19-nor and 5β,6β-epoxy derivatives

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*To gain further insight into the mechanism for inactivation of aromatase by androst-5-ene-7,17-dione (**1**) and its 19-nor analog **4**, 10β-oxygenated steroids **5** and **6**, $\Delta^{1(10)}$-steroid **7**, and 19-oxo-5β,6β-epoxy compound **8** were synthesized and tested for their ability to inhibit aromatase in human placental microsomes. All of the steroids studied inhibited the enzyme in a competitive manner with apparent K_i values ranging from 1.1 to 35 μM. The $\Delta^{1(10)}$-compound **7** was the most potent inhibitor among them. All of the inhibitors caused a time-dependent inactivation of aromatase in the presence of NADPH in air with the k_{inact} values ranging from 0.036 to 0.190 min^{-1}. The substrate androstenedione protected the inactivation, but a nucleophile, L-cysteine, did not, in each case. In contrast, each inhibitor did not cause the time-dependent inactivation in the absence of NADPH. These results show that the 5β,6β-epoxide **8** and/or the dienone **7** are not a reactive electrophile involved in the irreversible binding to the active site of aromatase during the mechanism-based inactivation caused by the suicide substrates **1** and/or **4**.* (*Steroids* **62**:516–522, 1997) © *1997 by Elsevier Science Inc.*

Keywords: aromatase; inhibitor; suicide substrate; time-dependent inactivation

Introduction

Aromatase is a cytochrome P-450 enzyme complex that catalyzes the conversion of androgens, androst-4-ene-3,17-dione (androstenedione), and testosterone to estrogens, estrone, and estradiol.[1–3] Aromatization of the androgens is thought to proceed with three sequential oxygenations at C-19 of the androgens, resulting in eventual less of the 19-angular methyl group and the elimination of the 1β,2β-hydrogens to form the estrogens. The first two occur at the C-19 position to produce 19-hydroxy and 19,19-*gem*-diol. Dehydration of this *gem*-diol leads to the readily isolated 19-oxo intermediate.[4–8] In the last step, C-19 and the 1β,2β-protons are eliminated as formic acid and water, respectively, to produce the estrogens[6–12] through either a direct fragmentation of 19-hydroxy-19-ferric peroxide intermediate with a six-membered transition state[6,13–15] or a Baeyer–Villiger reaction of the peroxide intermediate in two steps involving formation of 10β-formate followed by elimination of the 10β-formyloxy function.[16] The direct fragmentation sequence is currently thought to be likely.

Inhibitors of aromatase may be valuable as therapeutic agents in the treatment of estrogen-dependent tumors, including breast cancer.[17–20] We previously reported that C_{19} steroids having a unique α,β-unsaturated ketone, 4-en-6-one,[21] 5-en-4-one,[22] and 5-en-7-one **1**,[23] instead of a 4-en-3-one structure of the natural substrate androstenedione, along with 19-nor-5-en-7-one steroid **4**[24] (Figure 1) efficiently inhibit the aromatase activity in a competitive manner. Interestingly, only the 5-en-7-one steroids **1** and **4** among the conjugated ketones inactivate aromatase in a mechanism-based (suicide) manner. The 19-oxo derivative of inhibitor **1** also inactivates the enzyme in a suicide manner, suggesting that further oxygenation at C-19 would be involved in the aromatase inactivation by the inhibitor.[23,24] On the other hand, we recently established the mechanism for the aromatase inactivation caused by 6-oxoandrostenedione, one of the earliest discussed suicide substrates of aromatase;[25–27] 4β,5β-epoxy-19-oxo intermediate, which is possibly produced through the 19-hydroxy-19-hydroperoxide intermediate, alkylates a nucleophilic residue of amino acid of the active site of the enzyme.[28–30]

To gain further insight into the mechanism for the aromatase inactivation by the 5-en-7-one steroid **1** and its 19-nor analog **4**, we synthesized a series of the 19-nor derivatives (**5–7**) of compound **1** as well as 19-oxo-5β,6β-epoxy steroid **8**. All the steroids examined inhibited human placental aromatase in a competitive manner and also inac-

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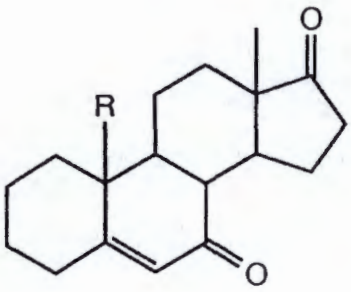

1: R=CH_3

2: R=CH_2OH

3: R=CHO

4: R=H

Figure 1 Structure of 5-en-7-one steroids.

tivated it in a time-dependent manner in the presence of NADPH, but did not in the absence of NADPH.

Experimental

Materials and general methods

Androst-5-ene-7,17-dione (**1**)[31] and its 19-hydroxy and 19-oxo derivatives **2** and **3**[23,24] were synthesized using the methods previously reported. [1β-^{3}H]androstenedione (27.5 Ci/mmol; ^{3}H-distribution, 74–79% at 1β) was obtained from New England Nuclear Corp. (Boston, Massachusetts, USA), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Kohjin Co. Ltd. (Tokyo, Japan).

Melting points were measured on a Yanagimoto melting point apparatus (Kyoto, Japan) and are uncorrected. Infrared (IR) spectra were recorded in KBr pellet on a Perkin–Elmer FT-IR 1725X spectrophotometer (Norwalk, Connecticut, USA), and ultraviolet (UV) spectra were determined in 95% ethanol on a Hitachi 150-20 UV spectrophotometer (Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$ solutions with JEOL EX 270 (270 MHz for ^{1}H and 67.8 MHz for ^{13}C) spectrometer (Tokyo, Japan) using tetramethylsilane as an internal standard. Mass spectra (MS) were determined with a JEOL JMS-DX 303 spectrometer. Thin-layer chromatography (TLC) was performed on E. Merck precoated silica gel plates (Darmstadt, Germany). Column chromatography was conducted with silica gel (E. Merck, 70–230 mesh) and a mixture of hexane and ethyl acetate as an eluent. High-performance liquid chromatography (HPLC) was carried out using a Waters 510 pump (Milford, Massachusetts, USA), Puresil C_{18} 5 μm 120 A column (150 mm × 4.6 mm i.d.; Waters), and a Waters 486 UV detector at 220 nm.

10β-Acetoxyestr-5-ene-7,17-dione (5)

A mixture of lead(IV) tetraacetic acid (1.06 g, 2.4 mmol), $CaCO_3$ (200 mg, 2.0 mmol), and cyclohexane (80 mL) was heated under reflux for 5 min; 19-hydroxyandrost-5-ene-7,17-dione (**2**) (230 mg, 0.76 mmol) was then added.[32] The reaction was carried out under reflux for 1 h. The mixture was then filtered through a bed of celite, which was washed with ethyl acetate. The filtrate was washed with NaI solution, $Na_2S_2O_3$ solution, and NaCl solution, sequentially, and dried with Na_2SO_4. Evaporation of the solvent gave an oil that was subjected to silica gel (30 g) column chromatography. The solid product obtained was recrystallized from acetone-hexane to yield compound **5** (180 mg, 72%). mp 134–136°C; FT-IR 1735 and 1673 (C=O) cm^{-1}; ultraviolet 238 nm (ε = 11,700). ^{1}H NMR δ 0.86 (3H, s, 18-Me), 2.15 (3H, s, 10β-OCOMe), 5.93 (1H, d, J = 1.3 Hz, 6-H); MS (*m/z*) (relat. int.) 330 (M^+, 10), 288 (15), 270 (45), 160 (25), 147 (100), 96 (30), 91 (20), 43 (35). Anal. calcd. for $C_{20}H_{26}O_4$: C, 72.70; H, 7.93. Found: C, 72.40; H, 7.77.

10β-Hydroxyestr-5-ene-7,17-dione (6)

K_2CO_3 (30 mg, 0.22 mmol) was added to a solution of the acetate **5** (30 mg, 0.09 mmol) in methanol (3 mL) and water (1 mL), and the mixture was heated under reflux in an N_2 atmosphere for 3 h. After this, the reaction mixture was concentrated to about 2 mL under an N_2 stream, diluted with ethyl acetate (50 mL), washed with water, and dried with Na_2SO_4. Evaporation of the solvent gave a solid that was purified by preparative TLC (hexane/ethyl acetate = 1:1, v/v) followed by recrystallization from acetone to yield compound **6** (18 mg, 70%). mp 212–213°C; FT-IR 3503 (OH) 1738 and 1657 (C=O) cm^{-1}; ultraviolet 232 nm (ε = 11,300). ^{1}H NMR δ 0.91 (3H, s, 18-Me), 5.74 (1H, d, J = 2.0 Hz, 6-H); MS(*m/z*) (relat. int.) 288 (M^+, 33), 270 (78), 242 (27), 226 (20), 214 (24), 160 (30), 147 (100), 91 (35), 79 (23), 41 (25). Anal. calcd. for $C_{18}H_{24}O_3$: C, 74.97; H, 8.39. Found: C, 74.70; H, 8.10.

Estra-1(10),5-diene-7,17-dione (7)

A solution of the 10β-acetate **5** (80 mg, 0.24 mmol) in N-methyl-2-pyrrolidone (1.6 mL) was heated at 160–180°C for 7 h. After cooling the reaction mixture to room temperature, the mixture was diluted with ethyl acetate (200 mL), washed with water, and dried with Na_2SO_4. After evaporation of the solvent, the resultant oily product was purified by preparative TLC (hexane/ethyl acetate = 2:1, v/v) followed by recrystallization from acetone to yield compound **7** (45 mg, 70%). mp 152–154°C; FT-IR 1735 and 1656 (C=O) cm^{-1}; ultraviolet 288 nm (ε = 14,600); ^{1}H NMR δ 0.90 (3H, s, 18-Me), 5.70 (1H, s, 6-H), 6.05 (1H, d, J = 4.0 Hz, 1-H); MS(*m/z*) (relat. int.) 270 (M^+, 53), 160 (25), 147 (100), 96 (32), 91 (20). Anal. calcd. for $C_{18}H_{22}O_2$: C, 79.96; H, 8.20. Found: C, 79.69; H, 8.30.

5β,6β-Epoxyandrosta-7,17,19-trione (8)

Aqueous 30% hydrogen peroxide (0.7 mL, 6.2 mmol) was added to a solution of the androst-5-ene-7,17,19-trione (**3**) (90 mg, 0.3 mmol) in methanol (10 mL), containing anhydrous $NaHCO_3$ (22 mg, 0.26 mmol), and the mixture was stirred at 0°C for 24 h,[33] diluted with ethyl acetate (100 mL), washed with $Na_2S_2O_3$ solution and water, and dried with Na_2SO_4. After evaporation of the solvent, a solid product was purified by silica gel (15 g) column chromatography followed by recrystallization from acetone to produce compound **8** (38 mg, 40%). mp 192–193°C; FT-IR 1733,1723, and 1715 (C=O) cm^{-1}; ^{1}H NMR δ 0.86 (3H, s, 18-Me), 3.29 (1H, s, 6α-H), 3.35 (1H, dd, J = 10.6 and 12.3 Hz, 8β-H), 10.02 (1H, d, J = 1.49 Hz, 19-H). ^{13}C NMR δ 13.9, 21.9, 22.2, 23.3, 24.2, 31.1, 34.2, 34.5, 35.3, 43.7, 44.1, 46.7, 51.8, 54.2, 61.9, 70.1, 202.7, 207.5, 218.9; MS (*m/z*) (relat. int.) 316 (M^+, 65), 287 (40), 259 (100), 241 (25), 177 (30), 149 (45), 135 (45), 121 (35), 105 (35), 91 (65), 79 (62), 67 (38), 55 (40), 41 (60). Anal. Calcd. for $C_{19}H_{24}O_4$: C, 72.13; H, 7.65. Found: C, 72.12; H, 7.79.

Preparation of placental microsomes

Human term placental microsomes (particles sedimenting at 105,000 g for 60 min) were obtained as described by Ryan.[34] They were washed once with 0.5 mM dithiothreitol solution, lyophilized, and stored at −20°C. No significant loss of aromatase activity occurred over the period of this study.

Aromatase assay procedure

Aromatase activity was measured according to the procedure of Siiteri and Thompson,[35] in which tritiated water released from [1β-^{3}H]androstenedione into the incubation medium during aromatization is used as an index of the enzyme activity. All were carried out in 67 mM phosphate buffer, pH 7.5, at a final incubation volume of 0.5 mL. The incubation mixture for the IC_{50} experiment contained 180 μM of NADPH, 0.3 μM of the ^{3}H-labeled substrate (3×10^5 dpm), 20 μg of protein of the lyophilized microsomes, various concentrations of inhibitors **5–8**, and 25 μL of methanol, and was incubated at 37°C for 20 min. For kinetic study, various concentrations of each of inhibitors **6** and **7**, 14.3, 19.0, 24.0, and 32.2 nM concentrations of the ^{3}H-labeled substrate, and a 5 min incubation time were used. Incubations were terminated by the addition of 3 mL of chloroform, followed by vortexing for 40 s. After centrifugation at 700 g for 5 min, aliquots (0.25 mL) were removed from the water layer and counted for determination of tritiated water production.

Time-dependent inactivation procedure

Various concentrations of inhibitors **5–8** were incubated with or without NADPH (600 μM), androstenedione (0.5 μM), and L-cysteine (0.5 mM) at 37°C with placental microsomes (200 μg protein) and methanol (25 μL) in 67 mM phosphate buffer, pH 7.5, in a total volume of 500 μL in air. Aliquots (50 μL), in duplicate, were removed at various time periods (0, 4, 8, and 12 min) and added to a solution of [1β-^{3}H]androstenedione (300 nM, 3×10^5 dpm) and NADPH (180 μM) in 67 mM phosphate buffer, pH 7.5 (total volume; 0.5 mL), and the mixture was incubated at 37°C for 20 min. The tritiated water release was determined as described above.

Reaction of the 5β,6β-epoxide 8 with N-acetyl-L-cysteine

A solution of the epoxide **8** (3.16 mg, 10 μmol), N-acetyl-L-cysteine (1.63 mg, 10 μmol), $NaHCO_3$ (1.68 mg, 20 μmol), in water (0.3 mL) and methanol or acetonitrile (1.2 mL) was shaken at 37°C. An aliquot (100 μL) of the reaction mixture was removed at an appropriate time and diluted with methanol or acetonitrile (100 μL). An aliquot (10 μL) of the diluted mixture was then subjected to HPLC. Amounts of the remaining epoxide **8** were obtained using absolute calibration method. HPLC conditions: solvent, methanol: water = 70:30 (v/v), 1.5 mL/min. Retention time: 3.0 min for **8**. The reaction was also analyzed by TLC. TLC conditions: solvent 1, hexane:ethyl acetate = 1:1 (v/v); solvent 2, $CHCl_3$:CH_3OH:HCOOH = 10:1:0.3 (by vol.). The R_f values of compound **8** were 0.73 (solvent 1) or 0.63 (solvent 2), respectively.

Results

Chemistry

Reaction of 19-hydroxyandrost-5-ene-7,17-dione (**2**) with lead(IV) tetraacetate gave the 10β-acetoxy derivative **5** in fair yield (Figure 2). Alkaline hydrolysis of the acetate **5** with K_2CO_3 in aqueous methanol afforded the 10β-hydroxy steroid **6**. In contrast, compound **5** was heated at 160–180°C in N-methyl-2-pyrrolidone to yield the 1(10),5-dien-7-one derivative **7** (70%). The ^{1}H NMR [δ 6.05 (1H, d, J = 4.0 Hz, 1-H)], IR (1656 cm^{-1}, a conjugated ketone), and ultraviolet (λ_{max} 288 nm) spectra of compound **7** are consistent with the assigned structure. Reaction of the 19-oxo-5-en-7-one steroid **3** with hydrogen peroxide in the presence of a weak base, $NaHCO_3$, gave the 5β,6β-epoxy derivative **8** (40%). In contrast, the 19-methyl compound **1** failed to react with hydrogen peroxide under similar conditions. These results correspond well to the previous results seen for the epoxidations of 19-oxoandrostenedione[33] and its 6-oxo analog,[30] indicating that compound **8** has the 5β,6β-epoxy ring. The spectra data and elemental analysis of the epoxides **8** were consistent with the assigned structure.

Biochemical properties

Inhibition of aromatase activity in human placental microsomes by the 19-nor (**5–7**) and 19-oxo-5β,6β-epoxy (**8**) steroids synthesized in this study, was examined in vitro by enzyme kinetics. The results are shown in Table 1. IC_{50}

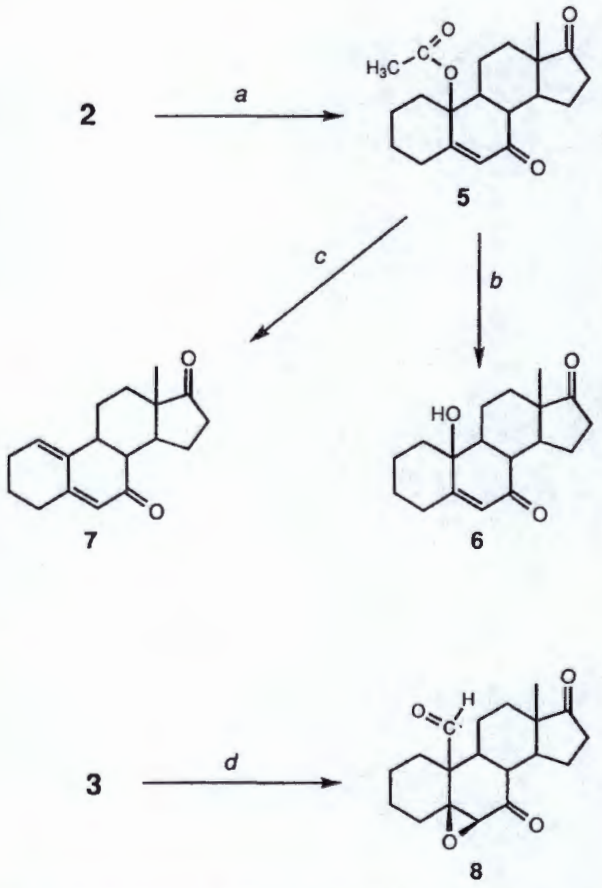

Figure 2 Synthesis of 19-nor and 5β,6β-epoxy steroid. Reagents: *a*, $Pb(OAc)_4$, $CaCO_3$, cyclohexane; *b*, K_2CO_3, MeOH, H_2O; *c*, N-methyl-2-pyrrolidone, 160–180°C; *d*, H_2O_2, $NaHCO_3$, MeOH.

values were initially obtained under initial velocity conditions, then to characterize the nature of inhibitor binding to the active site of aromatase, aromatization of androstenedione was measured at several inhibitor and substrate concentrations. The results of these studies were plotted on typical Lineweaver–Burk plots in which all the steroids studied exhibited clear-cut competitive inhibition. Analysis of Dixon plots gave the apparent K_i values ranging from 1.1–35 μM. The Lineweaver–Burk plot obtained by the dienone **7** is shown in Figure 3. In these studies, the apparent K_m for the substrate was found to be in the range of 30–35 nM.

Time-dependent inactivation of aromatase was observed when all of the inhibitors were incubated with aromatase in the presence of NADPH in air. Pseudo first-order kinetics were observed during the first 12 min of the incubation when kinetic data were analyzed according to Kitz and Wilson[36] (Figure 4A). Double-reciprocal plots of k_{obs} versus inhibitor concentration gave rate constants for inactivation (k_{inact}) and K_I values,[37] respectively, for all the inhibitors (Table 2). NADPH was essential for the time-dependent activity loss (Figure 4B) in all cases. The substrate androstenedione completely blocked the time-dependent inactivation, and a nucleophile, L-cysteine, had no significant effect on the inactivation in all cases (Figure 5).

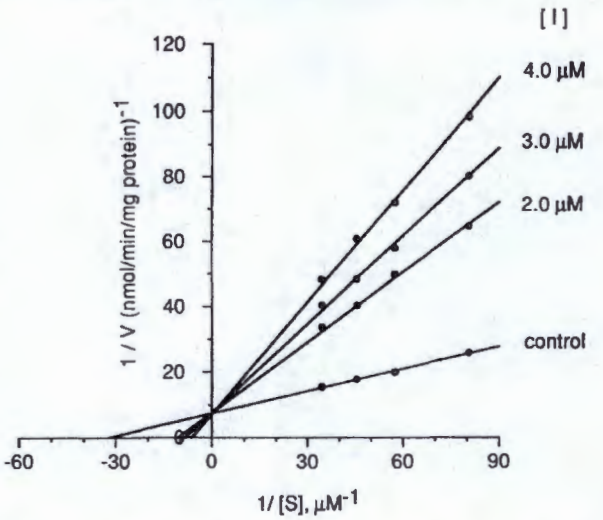

Figure 3 Lineweaver–Burk plot of inhibition of human placental aromatase by 1(10),5-dien-7-one steroid **7**. Each point represents the mean of two determinations that varied by less than 5% of the mean. The inhibition experiments with all the other steroids examined gave essentially similar plots to Figure 3 (data not shown).

Reaction of inhibitor 8 with N-acetyl-L-cysteine

To determine the chemical reactivity of inhibitor **8** toward a nucleophile, reaction of this compound with N-acetyl-L-cysteine in the presence of $NaHCO_3$ in aqueous methanol or aqueous acetonitrile was carried out, and disappearance of the inhibitor from the reaction mixture was monitored by HPLC. The inhibitor **8** disappeared in a time-dependent, pseudo first-order manner with a half-life ($t_{1/2}$) of 50 min in each experiment. Thin-layer chromatography (TLC) analysis of the reaction with compound **8** at 20, 40, and 60 min showed two spots corresponding to the substrate and a polar product [R_f: 0.73 and 0.00 (solvent 1) and 0.63 and 0.14 (solvent 2)].

Discussion

We synthesized three 19-nor steroids, the 10β-acetate **5**, the 10β-ol **6**, and the 1(10),5-diene **7**, as well as the 19-oxo-5β,6β-epoxide **8** and carried out a series of experiments to evaluate them as mechanism-based and active-site-directed irreversible inhibitors. All of the steroids studied were weak-to-poor competitive inhibitors of aromatase in human placental microsomes with apparent K_i values ranging from 1.1 to 35 μM. The dienone **7** was the most potent (K_i = 1.1 μM) among them, and its binding affinity to aromatase is similar to that (K_i = 2.4 μM) of the 19-nor steroid **4** and lower than that (K_i = 0.25 μM) of the 19-methyl steroid **1**. Introduction of an oxygen function (acetoxy or hydroxy group) at C-10β of compound **4** decreased the binding affinity to a great extent (K_i: 12 or 35 μM for the 10β-acetate **5** or the 10β-ol **6**). Based on these results along with the previous findings[23,24] regarding the binding affinities of the 19-oxygenated steroids **2** and **3** suggest that the 19-angular methyl would be essential for the tight binding of a 5-en-7-one steroid to the active site of aromatase. The binding affinity of the 19-oxo-5β,6β-epoxy compound **8** (K_i = 15 μM) was almost the same as that of the 5-ene compound **3**.

All of the inhibitors examined inactivated aromatase in a time-dependent manner with the k_{inact} ranging from 0.036 min^{-1} to 0.190 min^{-1} in the presence of NADPH in air. The apparent K_Is are similar to the apparent K_is obtained from the competitive experiments shown in Table 1 for each inhibitor, suggesting that the initial binding of the inhibitors to the enzyme is not rate-limiting in the inactivation process.

The rate of inactivation decreased when the substrate androstenedione was included in the incubation mixture. In the nucleophile protection experiment, L-cysteine failed to protect aromatase from inactivation by the inhibitors. Thus, covalent-bond formation between the enzyme and the reac-

Table 1 Aromatase inhibition by 19-nor and 5β,6β-epoxy steroids

Compound	IC_{50}, μM[a]	K_i, μM[b]
10β–Acetate **5**	92.0	12.0
10β–O1 **6**	310.0	35.0
1(10),5-Diene **7**	9.0	1.1
19-oxo-5β,6β-epoxy **8**	120.0	15.0

[a] [1β-^{3}H]Androstenedione, 300 nM; human placental microsomes, 20 μg protein; incubation time, 20 min.
[b] An apparent K_i value was obtained from a Dixon plot in which the apparent K_m for androstenedione was 33 nM. All of the compounds listed showed a competitive type of inhibition.
(c = 0)
(r = 3)

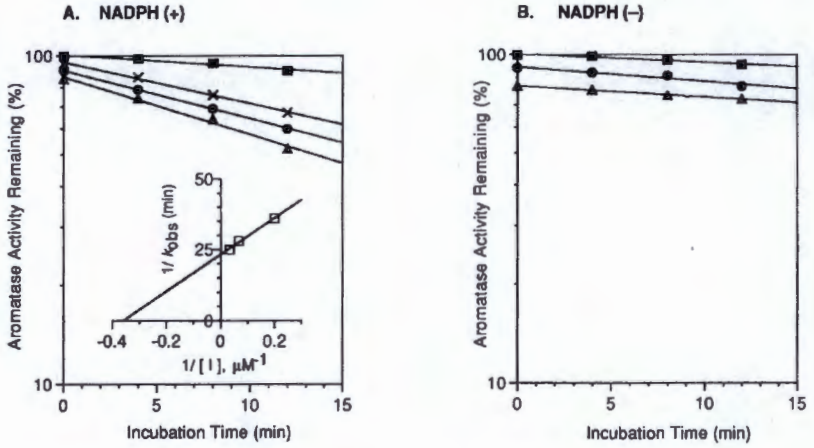

Figure 4 Time-dependent inactivation of human placental aromatase by 1(10),5-dien-7-one steroid **7** in the presence of NADPH (A) and in the absence of NADPH (B). Concentrations of the inhibitor; control (0 μM), ■; 5 μM, ×; 15 μM, ●; 30 μM, ▲. Each point represents the mean of two determinations that varied by less than 5% of the mean. The time-dependent experiments with compounds **5**, **6**, and **8** gave essentially similar plots to Figure 4 (data not shown).

Table 2 Kinetic analysis of time-dependent inactivation of aromatase caused by 19-nor and 5β,6β-epoxy steroids[a]

Compound	K_I, μM	k_{inact}, min^{-1}
10β–Acetate **5**	80.0	0.190
10β–O1 **6**	45.0	0.068
1(10),5-Diene **7**	3.0	0.044
19-oxo-5β,6β-epoxy **8**	12.0	0.036

[a] Apparent K_I and k_{inact} were obtained by Kitz-Wilson plot.
(c = 0)
(r = 3)

tive intermediate seems to occur rapidly at the active site; thereby, preventing diffusion of the activated inhibitor, a reactive electrophile, into the surrounding media.

The inactivation rate of the 10β-acetate **5** was faster than those of the 19-methyl steroid **1** and the 10β-ol **6** (k_{inact}: 0.190 versus 0.150 and 0.068 min^{-1} for **5** versus **1** and **6**). We previously reported that further oxygenation is required for the aromatase inactivation by the 19-oxo compound **3**, which is the oxygenated product of steroid **1**.[24] The acetate **5** is a homolog of the 10β-formyloxy compound, which is a product of the Baeyer–Villiger reaction of compound **3**. Although aromatization of androstenedione, a 4-en-3-one steroid, seems to proceed through the 19-hydroperoxide intermediate rather than the Baeyer–Villiger intermediate,[16] the faster inactivation rate for compound **5** suggests that the Baeyer–Villiger intermediate may, in part, be involved in the aromatase inactivation by the 5-en-7-one steroid **1**.

We previously predicted the 1(10),5-dien-7-one steroid **7** produced from the suicide substrates **1** and **4** during the course of the aromatase inactivation, would be a possible candidate for the reactive electrophile that binds to the active site of aromatase in a 1,6-addition manner.[23,24] Considering that both the 10β-oxygenated steroids **5** and **6** inactivate aromatase in a mechanism-based manner, this prediction seemed to be plausible. However, the 1(10),5-dienone **7** at concentrations of 30 and 60 μM did not cause the aromatase inactivation in a time-dependent manner in the absence of NADPH, while this caused the time-dependent inactivation in the presence of NADPH. The results indicate that the mechanism-based inactivation of aromatase by the parent steroids **1** and **4**, as well as the 10β-oxygenated steroids **5** and **6**, takes place not through the dienone intermediate **7**.

It has been reported previously that the aromatase inactivation by 6-oxoandrostenedione, a suicide substrate of aromatase, proceeds through its 19-oxo-4β,5β-epoxy derivative as a reactive electrophile irreversibly binding to the active site of aromatase.[30] Based on this, we focused on the 19-oxo-5β,6β-epoxide **8** as another candidate for a reactive electrophile involved in the aromatase inactivation by inhibitor **1**. This compound was chemically less reactive toward a nucleophile, L-cysteine, than the 19-oxo-4β,5β-epoxide ($t_{1/2}$: 50 min versus 40 s for **8** versus the other epoxide).[30] Furthermore, 5β,6β-epoxide **8** at concentrations of 30 and 60 μM did not inactivate aromatase in an affinity-labeling manner, indicating that steroid **8** is not involved in the inactivation by inhibitor **1**.

In conclusion, all of the inhibitors **5–8** studied inactivated aromatase in a suicide manner, but did not in an active-site-directed manner. On the basis of these results, it is concluded that the 5-en-7-one steroid **1** and its 19-nor analog **4**, suicide substrates of aromatase, inactivate the enzyme through a currently unknown reactive intermediate rather than the 1(10),-dienone **7** and/or the 19-oxo-5β,6β-epoxide **8**. However, it is presumed that the Baeyer–Villiger reaction of the 19-oxo intermediate **3** may be one of the possible inactivation sequences involved in the aromatase inactivation by the inhibitor **1**.

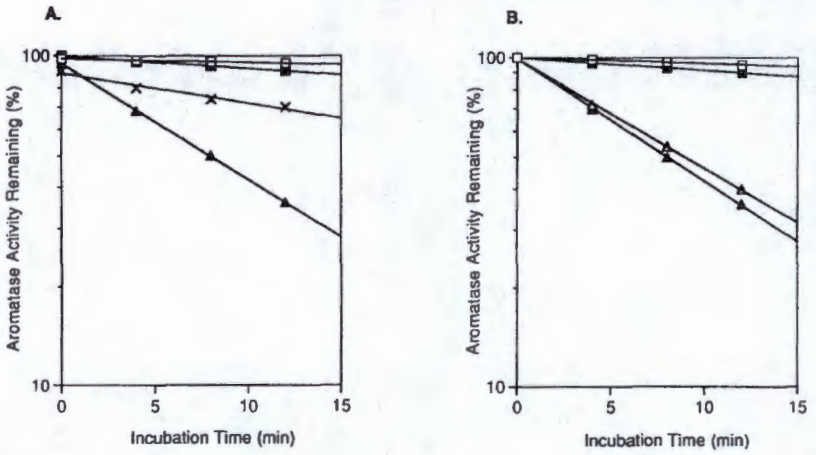

Figure 5 Inactivation of human placental aromatase by 10β-acetoxy steroid **5** under various conditions. (A) Androstenedione at a concentration of 0.5 μM (×) protected aromatase from inactivation caused by the inhibitor (70 μM) (▲) in the presence of NADPH. Control sample with (□) or without (■) androstenedione contained no inhibitor. (B) In the presence (△) or absence (▲) of L-cysteine (0.5 mM), a pseudo first-order inactivation of aromatase by the inhibitor (70 μM) was observed. Control sample with (□) or without (■) L-cysteine contained no inhibitor. Each point represents the mean of two determinations, which varied by less than 5% of the mean. The inactivation experiments with compounds **6–8** in the presence of androstenedione or L-cysteine gave essentially similar results to Figure 5.

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Androsta-3,5-diene-7,17-dione: Isolation from Urine and Formation from 7-Keto-dehydroepiandrosterone Sulphate under Various Conditions of Hydrolysis

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Androsta-3,5-diene-7,17-dione (5—7 μg per day) has been isolated from the urine of normal men. The following conclusions were made on the properties of 7-keto-dehydroepiandrosterone sulphate (3β-hydroxy-androst-5-ene-17-one-3-sulphate, sodium salt) under various conditions of hydrolysis (continuous ether extraction of acidified solution, enzymatic cleavage with the sulphatase containing preparation, acetic ester-solvolysis): 1. 7-keto-dehydroepiandro-sterone sulphate appeared to be a sulphate conjugate resistant to hydrolysis; 2. a complex hydrolysis may be achieved only at a high concentration of sulphuric acid. During this process one part of this compound is converted into androsta-3,5-diene-7,17-dione. With the aid of a sulphatase containing preparation a true hydrolysis without formation of artificial products takes place.

During the studies on the urinary excretion of α,β-unsaturated ketosteroids [Schubert, Wehrberger and Frankenberger 1964 a, b, c; Schubert and Wehrberger 1960, 1965; Schubert and Frankenberg 1968] small amounts of an unidentified compound were obtained. This substance appeared to be a part of a „weakly polar progesterone fraction" [Schubert, Wehrberger and Wachtel 1967] and after a detection according to Zimmermann, Anton and Pontius [1952] it gave a violet spot. Finally, the structure of this compound has been determined with the aid of UV- and IR-spectra of crystalline substance to be androsta-3,5-diene-7,17-dione. Because of its close relation to 7-keto-dehydroepiandrosterone (7-keto DHEA) and because of the extraction of androsta-3,5-diene-7,17-dione after a stepwise hydrolysis of urinary steroids [Schubert 1963] and under strongly acid reaction of the hydrolysis mixture, the cleavage of synthetic 7-keto-DHEA-sulphate and the formation of androsta-3,5-diene-7,17-dione under various conditions of hydrolysis has been studied.

Materials and Methods

Six liters of pooled urine obtained from 24hr collections from 5 healthy males aged 28—37 years were used. The steroid conjugates were subjected to stepwise hydrolysis [Schubert 1963]. The first step was the enzymatic splitting β-glucuronidase from Helix pomatia — RICHTER, Hungary 500 U/ml of urine, incubation 48 hr at 37 °C, the extraction of liberated steroids with ether) which was followed by a continuous ether extraction (24 hr) of the urine, acidified first to pH 1.0 with sulphuric acid and then adjusted to the final 4 N acid concentration. Following the washing with 1 N NaOH and water the extract was divided into a ketonic and non-ketonic fraction according to Girard [Zygmuntowicz et al. 1951]. The ketonic fraction was then chromatographed on the aluminium oxide (MERCK, 7.5% of water, 16 g, diameter of the column 11 mm) with the use of gradient elution (benzene/benzene — 1.33% ethanol, 76 fraction of 10 ml each). The fractions No. 1—18 were purified in Bush A system (benzine: methanol: water 100 : 85 : 15) on Schleicher -Schuell 2040 bm paper by ascending chromatography (44 cm). At 30—32 cm it was possible to find UV- and Zimmermann-positive substance. This was subjected to the two dimensional thin-layer chromatography on aluminium oxide D (VEB CHEMIEWERK GREIZ-DOLAU, German Democratic Republic) in benzene: ether 1 : 2, $R_F = 0.56$. Androsta-3,5-diene-7,17-dione which was prepared by dehydration of 7-keto-dehydroepiandrosterone acetate [Billeter and Miescher 1948] showed the same R_F value.

The UV-absorption spectrum showed the maximum at 279 nm which is typical for Δ3,5-diene-7-one. The infra-red spectrum (absorption at 1748, 1665, 1628 and 1598 cm^{-1}) was identical with that of authentic androsta-3,5-diene-7,17-dione. The melting point of the isolated material mixed with the authentic compound was 163—164 °C and did not show any depression. The amount of the isolated substance was about 5—7 μg per 24 hr urine.

Results

7-keto-dehydroepiandrosterone sulphate (sodium salt) was prepared by the exchange of 7-keto-DHEA with chlorosulphonic acid in pyridine according to Kornel et al. [1964]; melting point 163 — 165 °C [Baulieu and Emilozzi 1962].

For the estimation 1 mg the of sulphate conjugate (corresponding to 686/μg of 7-keto-DHEA or 645 μg of androsta-3,5-diene-7,17-dione was always taken and dissolved in 150 ml of water. The following hydrolysis conditions were used:

A, B: The solution was brought to pH 1.0 (A) with 70% sulphuric acid or to the final 4 N concentration of acid (B) and continuously extracted in a perforator for 24 hr.

C: To the aquaeous solution of the conjugate 22.5 ml of conc. hydrochloric acid were added and the mixtures was heated under reflux for 15 min. After cooling the residue was extracted 3 times with 50 ml of ether.

Table 1

The ratio of hydrolysis of 7-keto-dehydroepiandrosterone sulphate (expressed as the sum of 7-keto-DHEA and androsta-3,5-diene-7,17-dione) and the percentage of androsta-3,5-diene-7,17-dione in the mixture of liberated steroid under various conditions of hydrolysis
(the average of 4 parallel estimations)

Conditions of hydrolysis (see text)	Ratio of hydrolysis of 7-keto-DHEA sulphate	Percentage of androst-3,5-diene--7,17-dione
A	15%	20%
B	85%	25—30%
C	82%	95%
D	85%	3%
E	48%	10%
F	92%	25—30%

D: The solution of the conjugate in 150 ml of 0.1 M acetate buffer (pH 5.0) was incubated with the enzymic mixture from *Helix pomatia* (75 000 U) for 48 hr at 37 °C. The liberated steroids were extracted with ether.

E, F: 30 g of sodium chloride was added to the solution of the conjugate in 150 ml 0.1 N (E) or 2 N sulphuric acid (F) and the solutions were extracted subsequently with 300 ml and 150 ml of ethyl-acetate for 10 min each. The pooled extract E or F was maintained at 37 °C for 24 hr.

The ethereal extracts obtained according to the description A—D were washed with 1 N NaOH (2 times 1/10 of the total volume) and water (2 times 1/10 of the total volume), then dehydrated with sodium sulphate and evaporated to dryness. The ethyl acetate extracts E and F were washed with 1 N NaOH until the washing water showed the alkaline reaction. This procedure was followed by washing with water and evaporating under the reduced pressure.

Both androsta-3,5-diene-7,17-dione and 7-keto-DHEA were separated after the residue following the evaporation of the solvent had been dissolved in 5 ml benzene and put on the small Al_2O_3 column MERCK, prepared according to Brockmann, 1 g Al_2O_3, diameter of 5 mm). Following elution systems were used: 5 ml benzene, 10 ml 0.1% ethanol in benzene, 20 ml 0.5% ethanol in benzene (a), 20 ml 1% ethanol in benzene (b) and 6 ml of ethanol. Androsta-3,5-diene-7,17-dione was eluted in the Fraction a, whereas 7-keto-DHEA was in the Fraction b. The aliquot of each fraction was applied on the chromatographic paper and the effectiveness of the separation was esti-

7,17-dione is formed and that this formation depends on the reaction conditions used. The sulphate conjugate appears to be difficult to hydrolyze. During the continuous ether extraction of acidified water solutions (pH 1.0) during which DHEA-sulphate and androsterone-sulphate are continuously hydrolyzed, only a minute amount of conjugate is liberated. Further hydrolysis takes place only after further increase of the concentration of acid in the aqueous solution. Similar findings were described in a case of 16α-OH-DHEA [Okada et al. 1959; Schubert et al. 1962]. The percentage of the dehydration product androsta-3,5-diene-7,17-dione increased with the increased concentration of acid. During the acid hydrolysis at high temperature (boiling with HCl) only 3,5-diene-7,17-dione has been obtained. Further cleavage without any concomitant artifact formation has been obtained only after the addition of enzymic preparation from *Helix pomatia*. Similarly, the solvolysis in acetic acid ester showed an incomplete splitting of the conjugate at low acidity and an increased formation of 3,5-diene-7,17-dione under the increasing concentration of acid in aqueous solution. During these conditions of hydrolysis a minute amount of another artificial product has been found. This was a chlorine-containing compound which was not studied further.

The special circumstances of the behaviour of the sulphate conjugate during the hydrolytic process is to be taken into account during the estimation of 7-keto-DHEA. The enzymic preparations containing sulphatase activity are not commonly used and the solvolysis seems to be the most important wa to split the sulphate conjugate. The increased acid concentration during the solvolysis as well as the estimation of the formed andros androsta-3,5-diene-7,17-dione seem to be essential steps in the estimation of 7-keto-DHEA.

So far, the number of estimations of the secretion of 7-keto-DHEA by adrenals or of its urinary excretion is very small. These investigations could be of importance in certain pathological processes. Thus, in monkeys with haemorrhagic fever a remarkably increased concentration of 7-keto-DHEA in adrenal blood was found [Gontscharow et al. 1969].

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A Review of Dehydroepiandrosterone (DHEA)

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Abstract—Dehydroepiandrosterone (DHEA) is quantitatively the most abundant hormone in humans and mammals, with a wide variety of physiological effects, including major regulatory effects upon the immune system. Two of the most striking aspects of DHEA are a steady decline in DHEA with age and a significant deficiency in DHEA in patients with several major diseases, including cancer, atherosclerosis, and Alzheimer's disease.

The hormone is secreted in a non-sulfated (DHEA) and sulfated form (DHEA-S). The two are apparently interchangeable, and it appears likely that its physiological effects are achieved by derivative molecules that have yet to be identified.

DHEA Physiology

KEY PHYSIOLOGIC ASPECTS of DHEA are its involvement in antagonizing or counterbalancing the effects of glucocorticoids (Singh, 1994); its inhibition of glucose-6-phosphate dehydrogenase, the pentose shunt, ornithine decarboxylase, or K-channel blockade; as well as its inhibition of cytokines (Regelson, 1990a).

DHEA is hypolipidemic. Administration of DHEA leads to decreases in cholesterol, especially LDL (MacEwen, 1990).

DHEA is thought to have specific membrane receptors (Kalimi, Opoku et al., 1990). Perhaps the most striking aspect of DHEA is remarkable safety and freedom from side effects, even at doses of up to 4000 mg per day (Regelson, 1990b).

One of the many influences of DHEA is that of immune enhancement. DHEA appears to regulate, among other chemicals, Interleukin 2 synthesis (Daynes, 1990).

DHEA Deficiency

Several articles emphasize the decline in DHEA with age (Dilman, 1991; Orentreich, 1984; Ohashi, Kato et al., 1986; Malarkey, 1993; Barrett-Connor, 1986; Morales, 1994; Greenspan, 1991; Cacciari, 1990; Kalimi & Regelson, 1990) with only one known article stating that DHEA does not decline with age (Eggert-Kruse, 1994). Most articles state that 80-year-olds have only 10% of the levels of DHEA found in 25-year-olds. None of the articles reviewed rule out concomitant illnesses common with age, and none has been found in which extremely healthy 80-year-olds were studied. In our own clinic we have measured DHEA levels in approximately 1,000 individuals ranging in age from their mid-20s to their 90s. Even among a group of 50 nonpatient, healthy individuals, only 6 have had DHEA levels well above the laboratory means. Nichols Lab reports levels of 180–1250 ng/dl for men and 130–980 ng/dl for women. We cross-checked four different labs and only Nichols Lab had consistent results, with variations of less than 5%. Some labs varied by 100% on the same blood!

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Although most researchers do not distinguish between DHEA and DHEA-sulfate (DHEA-S) in either clinical laboratory significance or replacement therapy, there is some evidence that DHEA-sulfate is not as effective or reliable as DHEA (Jacobson, 1991; Ohashi, Kato et al., 1986; Ohashi, Fujio et al., 1986). It is generally believed that DHEA-S serves as a reservoir of weakly bound DHEA, but there is no clear proof that DHEA-S is fully metabolically available. For instance, in 108 seropositive HIV men with low levels of CD4 lymphocytes, DHEA levels were predictive of progression to AIDS but DHEA-S levels were not (Jacobson, 1991). With ACTH administration, DHEA increased only slightly in elderly adults, with even less increase in DHEA-S (Ohashi, Kato et al., 1986). With administration of CRF, DHEA levels increased 60% in young and old, but DHEA-S did not increase (Ohashi, Fujio et al., 1986).

Many authors reported low levels of DHEA in diabetes (Buffington, 1993; Nestler, 1992), coronary artery disease (Gordon, 1988; Barrett-Connor, 1986; Nestler, 1992; Barrett-Connor, 1990; Littman, 1993; Stahl, 1992; Ruiz Salmeron, 1992), various cancers (Giona, 1994; Bhatavdekar, 1994; Schwartz, 1993; Comstock, 1993; Bhatavdekar, 1992; Feo, 1990), obesity (Williams, 1993; Regelson, 1990a; Clearly, 1990), lupus (Kalimi & Regelson, 1990), hypertension (Shafagoj, 1992), AIDS (Wisniewski, 1993; Schinazi, 1990; Jacobson, 1991; Mulder, 1992), viral infections (Regelson, 1990a; Calabrese, 1990; Loria, 1990), Alzheimer's (Roberts & Fitten, 1990; Merril, 1990), and multiple sclerosis (Roberts & Fauble, 1990). DHEA appears to be a major modulator of the stress reaction, especially the increase of glucocorticoid (Singh, 1994; Dilman, 1992), and in animals DHEA is felt to be anxiolytic (Melchior, 1994).

DHEA Replacement

DHEA replacement therapy has been reported to be beneficial in a wide variety of illnesses. An increased sense of well-being and improved memory have been commonly reported (Morales, 1994; Roberts & Fitten, 1990; Bonnet, 1990; Merril, 1990). Improved insulin sensitivity and amelioration of diabetes have been reported with DHEA administration (Buffington, 1993). It has also been reported to assist in weight loss, but only in significantly obese individuals (Clearly, 1990).

Natural enhancement of DHEA has been reported with physical exercise (Cacciari, 1990), stress reduction programs (Littman, 1993), transcendental meditation (Glaser, 1992), and caloric restriction (Dilman, 1992; Turturro, 1991). DHEA also enhances the hypnotic and hypothermic effects of ethanol and pentobarbital (Melchior, 1992). Advanced angina was relieved by administration of only 80 mg DHEA-S/day.

Several U.S. patents have been issued for use of oral DHEA: #4,920,115 for reducing body fat mass, increasing muscle mass, lowering LDL cholesterol, and prevention of atherosclerosis; #5,110,810 and #5,162,198 for lowering platelet aggregation to reduce incidence of myocardial infarction and stroke; #4,835,147 for reducing symptoms of prostatic hypertrophy as well as nervousness of menopause, and decreased libido; #4,628,052 for treatment of rheumatoid arthritis, psoriasis, lupus, and other auto-immune diseases; and #4,518,595 for treatment of diabetes.

In summary, DHEA is not only the most prevalent hormone, it appears to be the most critical in predicting disease. It is deficient in every major disease, including obesity, diabetes, hypertension, cancer, immune deficiency, coronary artery disease, and various autoimmune disorders. Oral replacement of DHEA appears to be of benefit in all of these illnesses, with no reported complications. If natural enhancement of DHEA levels can be

achieved, such therapy may prove to be equally effective and theoretically more satisfactory in the long term.

Pertinent to this objective is the fact that Shealy and Myss have reported enhancement of DHEA blood levels by application of natural progesterone cream; the Liss Cranical Electrical Stimulator to 12 acupuncture points; or a very high frequency (52–78 Gigahertz) stimulator to these same points. Additive effects were seen with a combination of these techniques.

Conference on DHEA

On June 19 and 20 the New York Academy of Science hosted an international conference on DHEA. I will now review some of the findings.

The molecular structure of Dehydroepiandrosterone, $C19H28O_2$, has been known for at least twenty years. Secreted by the adrenal cortex, it was thought to be a supplementary androgenic hormone, synthesized in the body from cholesterol and secreted in urine by both men and women. A sulfated version (DHEA-S) has also been recognized, and it was known that the two forms of the molecule could change back and forth. During the past ten years it has been learned that DHEA is normally the principal secretion of the adrenal cortex, except during stress or serious illness, when cortisol secretion predominates. Recently, it has been learned that DHEA-S's metabolic function is not so much androgenic as it is a very important regulator of metabolism, in partnership with melatonin and other metabolic regulators. (Pierpaoli et al., 1994). In essence it is a glucocorticoid antagonist. It may bind to the glucocorticoid receptor or, more likely, down regulate glucocorticoid receptors.

It is now known that the secretion of both hormones decreases with aging, and there is evidence from rat experiments that, if administered, these hormones may prolong the normal life span of rats.

There is published evidence of the following physiological effects of DHEA, reflecting its modulating effect on cortisol and as an antagonist of GABA. Clinically DHEA has antidiabetic effects. Administration of DHEA has reduced insulin requirements in diabetic patients. It also has antiobesity effects, diminishing food intake; antiviral and antibacterial effects; and antiaging effects have also been reported (Majewska, 1995).

Among the multitude of actions of this long neglected hormone are its antioxidant and anticancer effects (Pentti et al., 1995).

DHEA is widespread in the brain, the highest uptake of exogenous DHEA being the pineal within an hour of administration. It is also taken up by amygdala, hippocampus, thalamus, midbrain, and frontal cortex. DHEA is very low compared to cortisol in Alzheimer patients. This is also true in depressed patients. DHEA has been shown in animals to bolster thymocyte production, and it counteracts thymus involution. Improvement in immune function in men has also been reported (Yen, 1995).

DHEA was reported to act as an antidepressant in humans and to improve memory and cognition in humans. Evidence of DHEA-S capability to protect against coronary atherosclerosis was adduced from studies of human subjects (Herrington, 1995; Jesse, 1995).

The multitude of metabolic effects of DHEA appear to be achieved by various derivative molecules. The effects of some of these have been studied by Lardy (Lardy, 1995).

Severe physical stress affected DHEA concentration in healthy army rangers, elevating serum cortisol and decreasing DHEA/cortisol ratio (Bernton et al., 1995).

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The Enzymic Hydrolysis of Steroid Conjugates

2. HYDROLYSIS OF STEROID CONJUGATES IN URINE

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That urinary androgenic substances might be excreted as conjugates was first indicated by Funk, Harrow & Lewja (1929). Since that date, only two types of steroid conjugate have been isolated from urine—the glucuronides and the sulphates.

It has long been recognized that the drastic conditions of acid hydrolysis necessary to release steroids from these water-soluble derivatives in urine may result in losses or chemical transformations. Mild forms of acid hydrolysis have also been tried, but those steroids linked with glucuronic acid require for hydrolysis the stronger acid conditions which are responsible for the production of artifacts. These problems have been reviewed by Pincus (1954), and by Birke & Plantin (1954).

The successful use of bacterial glucuronidase for the hydrolysis of steroid glucuronides was reported by Buehler, Katzman, Doisy & Doisy (1949). Several authors (e.g. Cohen, 1951; Corcoran, Page & Duston, 1950; Cox & Marrian, 1951; Kinsella, Doisy & Glick, 1950) have since demonstrated that hydrolysis of urinary steroid conjugates with preparations of β-glucuronidase liberated considerably more formaldehyde-producing material from urine than hydrolysis and extraction at pH 1. More recently, Katzman, Straw, Buehler & Doisy (1954) have described the action of bacterial β-glucuronidase preparations on urinary oestrogen conjugates. A study of the hydrolysis of steroid conjugates by calf-spleen β-glucuronidase was made by Cohen (1951). Bitman & Cohen (1951) compared the hydrolysis of conjugated 17-oxosteroids by the acetate-buffer technique of Talbot, Ryan & Wolfe (1943), by calf-spleen β-glucuronidase and by hydrochloric acid at pH 1.

This paper describes the application of sulphatase and β-glucuronidase of molluscan origin to the hydrolysis of some urinary neutral 17-oxosteroid sulphates and glucuronides.

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EXPERIMENTAL

The enzyme powders were prepared from specimens of *Patella vulgata* by the procedure described in the previous paper (Stitch, Halkerston & Hillman, 1956). The sulphatase activity of the powders used varied between 2000 and 15 000 units/g., and had an average β-glucuronidase activity of $1{\cdot}5 \times 10^6$ units/g. A powder containing 9100 units of sulphatase/g. and $1{\cdot}2 \times 10^6$ units of β-glucuronidase/g. was used for the study of the effect of pH, enzyme concentration, time and inhibitors on the enzymic process in urine.

Enzymic hydrolysis, extraction and estimation of urinary neutral 17-oxosteroids. In the following enzyme experiments, the urine samples were warmed to 37° in small flasks and adjusted to the required pH. An aqueous solution of the enzyme was added and the pH finally checked. The flasks were lightly stoppered with cotton wool and incubated at $37 \pm 0{\cdot}1°$ for the required time. The samples were then cooled and extracted four times with equal volumes of ether. The combined ether extracts were washed twice with 2N-NaOH (¼ vol.), and finally three times with distilled water (¼ vol.). Troublesome emulsions, when encountered, were broken by centrifuging. The washed ether extracts were dried over sodium sulphate for 3–4 hr. and filtered through a sintered-glass funnel (1A), under reduced pressure. The ether was evaporated and the residue dissolved in ethanol. Suitable portions were taken for the estimation of the 17-oxosteroids, by means of the Zimmermann reaction.

Acid hydrolysis, extraction and estimation of urinary neutral 17-oxosteroids. The urine samples were refluxed with concentrated HCl (15 ml./100 ml. of urine) and CCl_4 (20 ml./100 ml. of urine) according to the method of Callow, Callow, Emmens & Stroud (1939). The combined CCl_4 extracts were washed twice with 2N-NaOH (¼ vol.) to remove phenolic substances, and three times with distilled water (¼ vol.). The washed extract was dried over Na_2SO_4 for 3–4 hr., filtered through a sintered funnel and concentrated by distillation; it was then made up to 50 ml. with CCl_4. Suitable portions were evaporated to dryness at 100–110° (oil bath), and the 17-oxosteroid content was estimated by means of the Zimmermann reaction (Callow, Callow & Emmens, 1938).

Partition chromatography. Fractionation of the extracted 17-oxosteroids was carried out by partition chromatography on columns of silicic acid with nitromethane as stationary phase, and light petroleum (boiling range 60–80°) containing 3% of $CHCl_3$ as mobile phase (Jones & Stitch, 1953).

RESULTS

The effect of pH (3·55–5·5) on the rate of hydrolysis of the urinary 17-oxosteroid conjugates showed a shallow pH optimum in the region of 4·5–5·0. The rate of hydrolysis increased rapidly with increase in enzyme concentration up to about 50 mg. (approximately 450 units of sulphatase and $6{\cdot}0 \times 10^4$ units of β-glucuronidase/25 ml. of urine), but further increase had little effect.

Rate of release of free 17-oxosteroids from urine by an extract of P. vulgata

The enzymic hydrolysis of 25 ml. portions of urine was studied over a period of 140 hr. After extraction of the 17-oxosteroids, the residual urine was hydrolysed with acid in order to obtain an estimate of the amounts of conjugated 17-oxosteroids remaining after enzymic hydrolysis. Untreated urine controls were incubated at the same pH. Results shown in Fig. 1 reveal an increase in 17-oxosteroids with time up to 70 hr., together with decreased residual 17-oxosteroid.

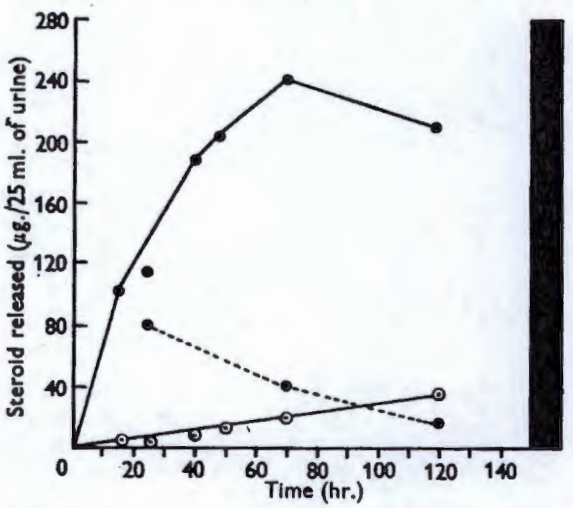

Fig. 1. Release of 17-oxosteroids from urine by enzyme from *P. vulgata*. Portions (25 ml.) of a 24 hr. urine were incubated at $37° \pm 0{\cdot}1°$ with 450 units of sulphatase and 6×10^4 units of β-glucuronidase in 50 mg. of powder. The pH was adjusted to 5·0 with acetate buffer. Controls without enzyme were incubated at the same pH for the same periods. Residual oxosteroid remaining after enzymic hydrolysis was determined by acid hydrolysis. The total 17-oxosteroids released by acid hydrolysis of a 25 ml. portion is shown by the height of the black area. ●—●, Limpet extract; ●- -●, residual ketosteroid released by acid after enzymic hydrolysis; ⊙—⊙, untreated urine.

Removal of inhibiting ions from solution before enzymic hydrolysis of the urinary 17-oxosteroids

Sulphate. Normal male urine samples (25 ml.) were treated with 2 ml. of an aqueous solution of barium chloride saturated at room temperature (18°). The precipitated barium sulphate was left in suspension. Portions (25 ml.) of the same urine similarly treated gave no further precipitate when centrifuged and treated with additional barium chloride. The pH of each portion was adjusted to 5·0 with N-HCl, and 450 units of sulphatase and 6×10^4 units of β-glucuronidase in 50 mg. of powder were added. Incubation was carried out at 37° for periods of time varying from 5 to 96 hr. The samples were extracted and the 17-oxosteroid content was estimated by the Zimmermann reaction. Untreated urine controls were incubated at the same pH. The time–hydrolysis curves which were obtained indicated a slight acceleration of the

hydrolysis rate after precipitation of the sulphate ion (Fig. 2).

In a similar experiment on another urine sample, the precipitated barium sulphate was removed by filtration before incubation. The results again showed that the effect of sulphate removal was a slight (12%) increase in the rate of hydrolysis. Barium has been shown to be without appreciable effect on the enzymic hydrolysis of dehydro*epi*-androsterone sulphate and of phenolphthalein β-glucuronide (Stitch *et al.* 1956).

Sulphate and phosphate. Portions (25 ml.) of a normal male urine were adjusted to pH 11·5 with 2N-NaOH, treated with 2 ml. of a 10% aqueous solution of barium chloride and centrifuged, and the pH of the supernatant urine was adjusted to 5·0 with HCl. A total of 450 units of sulphatase and 6×10^4 units of β-glucuronidase in 50 mg. of powder was added and incubation carried out for 2 hr. at 37°. Treatment of similar urine samples with additional barium chloride after centrifuging gave no further precipitate. The results indicated a four-fold increase in the rate of hydrolysis over that of the controls which were not treated with barium chloride.

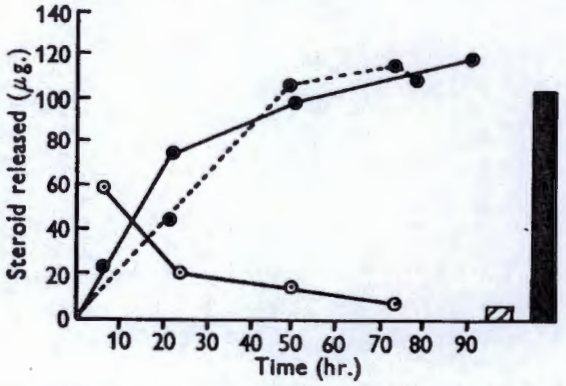

Fig. 2. Effect of removal of sulphate ion on rate of hydrolysis of urinary 17-oxosteroids. Portions (25 ml.) of 24 hr. urine were incubated at 37° ± 0·1° with 450 units of sulphatase and 6×10^4 units of β-glucuronidase in 50 mg. of powder at pH 5·0; 25 ml. portions of the same urine were incubated, after precipitation of sulphate, under the same conditions. The total oxosteroids released by acid hydrolysis is shown by the height of the black area. The shaded area shows the untreated urine control incubated at the same pH; ●—●, effect of enzyme after precipitation of sulphate; ●- - -●, effect of enzyme without precipitation of sulphate; ⊙—⊙, residual oxosteroid released by acid after enzymic hydrolysis.

Table 1. *Comparison of the total neutral 17-oxosteroids released by acid and by enzyme: hydrolysis of 250 ml. portions of different urines*

Sample of urine	17-Oxosteroids (mg./24 hr.) Acid hydrolysis	Enzyme hydrolysis	$\frac{\text{Enzyme}}{\text{Acid}} \times 100$
Normal (pooled male)	5·2*	5·0*	96·1
Mental patients (male)			
1	11·4	10·0	87·8
2	13·7	11·2	81·9
3	13·7	12·5	91·3
4	6·0	6·4	106·5
5	9·7	11·7	120·5
6	8·7	9·05	109·0
7	9·8	11·05	112·0
8	7·5	8·95	120·0
9	15·45	17·2	111·1
10	7·2	6·8	94·5
11	13·4	13·4	100·0
12	18·6	18·2	98·0
13	10·5	9·75	93·0
14	23·2	13·9	60·0
15	5·15	5·3	102·5
16	7·5	3·35	44·4
Mental patients (female)			
1	9·8	8·9	90·9
2	2·85	2·4	83·1
3	2·3	1·6	69·4
4	9·1	8·1	89·0
5	8·5	5·1	59·8
6	12·3	11·8	96·0

* mg./l.

Effect of sulphate and phosphate precipitation on the 17-oxosteroid content of urine

Two 25 ml. portions of urine were treated with barium chloride as described in the previous experiment. In this case the precipitated barium salts were washed with 3 ml. portions of water and the washings added to the supernatant urine. As controls, two further 25 ml. portions of urine were treated similarly but with distilled water replacing the barium chloride solution. All four samples were then subjected to acid hydrolysis in the presence of carbon tetrachloride, and the 17-oxosteroid content of the carbon tetrachloride extracts was determined as described above. The results indicated that a significant (16%) loss of 17-oxosteroid occurred during the precipitation of the sulphate and phosphate ions. Attempts to extract the adsorbed 17-oxosteroid from the precipitated barium salts included extraction with two 5 ml. portions of ethanol followed by acid hydrolysis (in the presence of carbon tetrachloride) of the residue. The results showed that in neither case could any 17-oxosteroid be recovered.

Comparison of acid and enzymic hydrolysis

In this series of experiments, carried out by Dr J. J. Gordon, urines from normal males and mental patients were taken for a comparison of the amounts of 17-oxosteroids released by either acid or enzymic hydrolysis.

One sample of each urine was hydrolysed with acid as described above and the second was adjusted to pH 4·5 with HCl, and an amount of enzyme containing 5000 units of sulphatase and approx. $0{\cdot}75 \times 10^6$ units of β-glucuronidase was added.

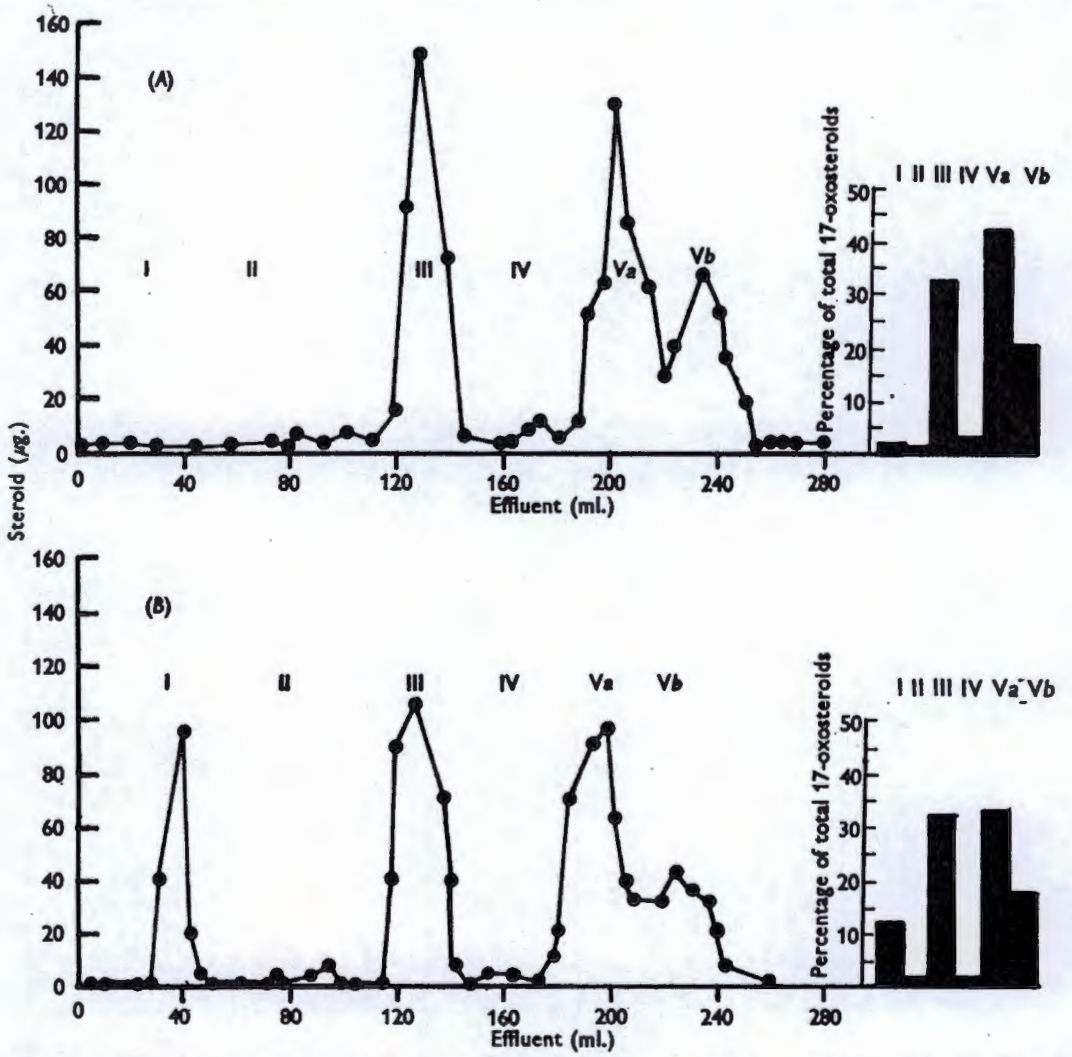

Fig. 3. Partition chromatography of 17-oxosteroid extracts from normal male urines: (*A*) after enzymic hydrolysis; (*B*) after acid hydrolysis. The following steroids are eluted in the peaks: I, artifacts (chiefly 3β-chloroandrost-5-en-17-one); II, unknown; III, 3:5-*cyclo*androstan-6-ol-17-one and androsterone; IV, androstan-3β-ol-17-one; V*a* and V*b*, androst-5-en-3β-ol-17-one; testan-3α-ol-17-one.

Incubation was carried out at 37° for 96 hr. The results of this comparison are shown in Table 1 and have been calculated in terms of androst-5-en-3β-ol-17-one after correction for non-ketonic chromogens by the method of Cook & Rooks (1952).

Partition-chromatographic analysis of the 17-oxosteroids extracted from urine after enzymic or acid hydrolysis

Two 24 hr. specimens of normal male urine were divided equally; one portion of each specimen was subjected to acid hydrolysis and the other incubated at pH 5·0 with sufficient enzyme to give 20 000 units of sulphatase/l. of urine and approx. $3{\cdot}0 \times 10^6$ units of β-glucuronidase/l. Incubation was carried out at 37° for 96 hr. After incubation the liberated steroids were extracted and washed. A portion of the total extract containing 3–4 mg. of total 17-oxosteroid was evaporated to dryness, and taken for chromatography. Fractionation was carried out as described by Jones & Stitch (1953). The chromatographic patterns obtained are illustrated in Fig. 3. These show a difference in the distribution of the peaks according to the method of hydrolysis employed.

In some cases a rather different elution pattern was obtained which seemed to indicate formation of additional polar steroids during enzymic hydrolysis.

DISCUSSION

The results described above show that the molluscan-enzyme preparations are capable of releasing some urinary neutral 17-oxosteroids from their water-soluble derivatives in urine. The comparison between the enzymic hydrolysis and the conventional acid hydrolysis shows some similarity in total 17-oxosteroid values obtained. However, on fractionation of the steroid extracts it is clear that the compounds generally accepted as artifacts of acid hydrolysis (Fig. 3, peak 1) are absent from the extract derived after enzymic hydrolysis. Furthermore, it appears that other peaks are increased when the enzyme extract is employed. It is noted that in spite of the failure of the enzyme extract to hydrolyse pure androsterone sulphate (Stitch *et al.* 1956) the chromatographic peak corresponding to the position of androsterone in the elution sequence (peak 3) was obtained in similar yield by both acid and enzymic hydrolysis. This would appear to indicate that the androsterone was not excreted, in the urine samples studied, in the sulphate form or that hydrolysis of this compound occurred by a mechanism other than that initiated by the added enzyme. Recently, Jayle & Baulieu (1952) found androsterone only in the fraction soluble in butanol at pH 11 but not hydrolysed at pH 5·8. However, Birke & Plantin (1954) found that androsterone comprised 20% of the liberated steroids after hydrolysis of the urinary conjugates with 1:4-dioxan and chromatography and infrared spectroscopy of the hydrolysate.

Decomposition of the 17-oxosteroid by bacteria may account for the decline in steroid value observed after prolonged incubation (Fig. 1). This fall in value was also observed on butanol extracts of urine but was not a general occurrence. Attempts to prevent bacterial action by the addition of penicillin G (5000 units) and streptomycin sulphate (2·5 mg.) (without effect on the enzymic-hydrolysis rate of dehydro*epi*androsterone sulphate and phenolphthalein β-glucuronide), to a butanol extract (5 ml.) of 50 ml. of urine, gave inconclusive results since control urines and urines with added antibiotic showed similar rates of hydrolysis, and neither showed the late fall in 17-oxosteroid value. A small amount of residual 17-oxosteroid always appears to remain unhydrolysed after enzymic hydrolysis has been carried out (Figs. 1, 2).

The variable results for the comparison of steroid released by acid and by enzymic hydrolysis can be accounted for by the high but variable concentration of phosphate inhibitor in urine and by difficulties in the extraction of the steroids from urine after enzymic hydrolysis.

Although this enzymic procedure has been shown to reduce the artifact normally associated with acid hydrolysis, no account has been taken of possible steroid transformations which may be effected by enzyme systems contained in the urine or in the added molluscan extracts. Such effects have been demonstrated recently (Talalay & Bobson, 1953). It is significant that microbiological hydroxylation of many steroids has recently been reported by several authors, e.g. Zaffaroni, Campillo, Cordoba & Rosenkranz (1955). Enzyme systems in the urine could have been destroyed by boiling, but this was felt to be undesirable because of the possible effects of heat on the 17-oxosteroids. The negligible blanks obtained indicated that endogenous β-glucuronidase and sulphatase were not responsible for the hydrolysis of the steroid conjugates. In view of the possibility of steroid transformation by unknown enzyme systems, the extracts obtained after this form of hydrolysis cannot necessarily be considered free from artifact.

The marked specificity of the sulphatase and the relatively high release of 17-oxosteroid from urine on occasions suggests that sulphates other than those of the 3β-, 5α- and Δ^5-series may comprise a small fraction of the total 17-oxosteroids, or that hydrolysis of these conjugates may be effected by some other process.

SUMMARY

1. The application of a sulphatase and β-glucuronidase extract of molluscan origin to the hydrolysis of urinary steroid conjugates has been described.

2. The effect of varying conditions of pH, time and enzyme concentration on the rate of hydrolysis of the conjugates has been studied.

3. The influence of some urinary inorganic inhibitors on the rate of enzymic hydrolysis has been indicated.

4. A comparison of the amount of neutral 17-oxosteroids released by the methods of enzymic and acid hydrolysis has been made.

5. Partition chromatography of urinary neutral 17-oxosteroids after both enzymic and acid hydrolysis has been carried out. A difference in the distribution of various chromatographic fractions depending on the method of hydrolysis was noted.

The authors wish to thank Dr J. J. Gordon for supervising the comparison of 17-oxosteroids released by enzymic and by acid hydrolysis.

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The Selective Solvation of the Hyaluronic Acid Complex of Ox Synovial Fluid

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This work was undertaken to test the suggestion made by Johnston (1955) that a large part of the volume occupied in solution by the coiled-chain particles of the hyaluronic acid complex (Ogston & Stanier, 1951, 1952) might be 'impenetrable' by serum albumin. Such an impenetrability could equally be regarded as a positive selective solvation of the hyaluronic acid complex by components of the solution (water and buffer salts) other than albumin (Ogston, 1954). This hypothesis was tested by studying the effect of concentration of albumin on the sedimentation of the hyaluronic acid complex in the ultracentrifuge; this is the method used by Lauffer and his colleagues (see Lauffer, Price & Petre, 1949) to estimate the 'solvation' of virus particles.

The rate of sedimentation of the hyaluronic acid complex becomes zero when the reciprocal of its effective specific volume (i.e. its effective density in solution) is equal to the density of the solution. Its effective density can be regarded as being determined by the relative proportions in the sedimenting particles of (i) unsolvated hyaluronic acid complex, (ii) solvent free of albumin, (iii) solvent containing albumin, of the same composition as that of the bulk solution.

EXPERIMENTAL

Hyaluronic acid complex. This was prepared by the method of Ogston & Stanier (1950). The complex was washed 16 times with buffer on a sintered-glass filter of average pore diameter 1 μ. The resulting solution showed only a single, very sharp boundary in the ultracentrifuge, and no sign of any free protein.

* Fellow of the Arthritis and Rheumatism Foundation.

Original Article

Influence of Polluted SY River on Child Growth and Sex Hormones*

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Abstract

Objective To investigate the influence of the polluted SY River on children's growth and sex hormones, and provide scientific data for assessment of the polluted status of the SY River.

Methods The study areas were selected randomly from the SY River Basin. Lead (Pb), mercury (Hg), arsenic (As), phthalates (DEP, DBP, DMP, DEHP), and bisphenol A (BPA) were measured both in the river water and in the drinking water. School children were selected by cluster sampling (n=154). Physical development indexes (height, weight, bust-circumference, and skinfold thickness) and sex hormones [testosterone (T) and estradiol (E2)] were measured for all the children.

Results The contents of Pb and Hg exceeded Class V standards of surface water quality in each section of the river and other indicators exceeded Class III. Compared to the control area, the concentrations of Pb, Hg, As, BPA, DEP, and DBP in the drinking water were significantly higher than in the polluted area (P<0.05). Children from the control area had significantly lower E2 and T than children from the polluted area (P<0.05). Among anthropometric results, only skinfold thickness had statistically significant difference between the two groups (P<0.05), while the other indexes showed no significant differences between the two groups (P>0.05).

Conclusion The drinking water has been polluted by the SY River and affected serum sex hormone levels of children living in the polluted area.

Key words: Water pollution; Children; Growth and development; Estradiol; Testosterone

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INTRODUCTION

The Huai River is the third largest river in China, which runs through Henan, Anhui, Shandong, and Jiangsu provinces. It has a total length of 1 000 km and covers an area of 270 000 km^2. About 150 million people live in the Huai River Basin. With population growth, economic development and rapid acceleration of urbanization since the 1980s, domestic sewage, industrial effluents and agricultural pollutants have been poured into the river, making the water quality increasingly worse.

The SY River is located in the hinterland of Henan Province and is the largest tributary to the Huai River. With a total length of 418 km, it runs through the cities of Pingdingshan, Yuzhou, Xuchang, Luohe, and Zhoukou, and covers an area of 34 400 km^2. Various types of wastewater from 31 cities flow into the river without any sewage disposal.

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Some researches showed that the SY River had lost its self-purification capacity because of receiving so much municipal and agro-industrial wastewater[1-3]. The SY River is one of the most polluted rivers in the Huai River Basin with water quality classified as Class V or worse[2]. According to the monitoring data, a variety of harmful pollutants have been detected in the groundwater near the SY River at a depth of 20-50 m or deeper[4]. Previous studies showed that water pollution could cause health damage such as increasing incidences of cancer[5] and that the heavy metal contaminants in water could damage the body's nervous system and interfere with the endocrine system even at a low concentration[6].

The effects of the Huai River pollution on health of local people are a major issue of social concern, which have been reported by many studies[7-8]. According to one study, the incidence of malaria was connected with the distance from the river and 74% of malaria cases were inhabited within the extent of 60 m near the Huai River[7]. Another study revealed that the pollution level of the Huai River water had a significant positive correlation with the incidence of gastric cancer, liver cancer, esophageal cancer, and other malignant tumors among local people[8]. However, most of the observations have paid more attention to the adult population, and health researches on children and adolescents are rarely reported. As children are in a critical development period, especially those aged 8-13 years , they are more sensitive to environmental pollution than adults. The groundwater was one of the most important sources of drinking water to the local people, and there were no other sources of pollution in the two survey areas in our study. Therefore, the objective of this study was to evaluate the polluted status of the SY River and the drinking water and investigate the influence of water pollution on children's health, which would provide a scientific basis for selecting monitoring indicators.

MATERIALS AND METHODS

Location

The cross sectional study was conducted in S County which is the last county that the SY River runs through in Henan Province. The village located less than 2 km and the other one located more than 20 km away from the SY River Basin were randomly selected as a polluted and control area, respectively. The two villages were similar in the economic level, demographic composition and living habits.

Subjects

Children aged 8 to 13 years old who were born and lived in the polluted and control areas were selected by cluster sampling. They were all the primary school students (n=154, 73 boys and 81 girls). 69 of them were from the polluted area (30 boys and 39 girls) and 85 from the control area (43 boys and 42 girls).

Sampling and Determination of Water Pollutants

The total length of the SY River is 32.5 km in S County. Three sampling sections were set up at S County. The upstream section (US) was set up at the place where the SY River just enters the county, the midstream section (MS) at the Huaidian sluice gate and the downstream section (DS) at the place where the SY River runs out of the town. Three samples were collected from each section by quartering, and each water sample was measured three times. Drinking water samples were collected from five positions (east, south, west, north and central) of two villages, respectively, and three samples were collected from each position and each sample was measured two times.

The concentrations of pollutants including Pb, Hg, As, Bisphenol A (BPA) and dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and dioctyl phthalate (DEHP) were measured for both the river water and the drinking water. All indicators were tested in accordance with the monitoring and analytic methods for surface water and wastewater[9].

Questionnaire

The questionnaire included children's birth and feeding patterns, birth weight, maternal education, passive smoking status, the number of days in a week for breakfast, exercise time, sleep time, the pesticide exposure of the mother during pregnancy etc.. Investigators were trained in advance and parents of students were visited by face to face interview .

Anthropometric Measurement

Participants' weight, height, bust circumference and skinfold thickness were measured with underwear only and without shoes. Body weight was

measured with a digital lithium weighing scale, height with a portable height and sitting height meter and bust circumference with a Gulick anthropometric tape. Skinfold thickness on triceps was obtained by using a Harpenden caliper.

Sex Hormone Analyses

The subjects were requested to fast for at least 10 hours before they came for the study. The fasting blood samples, totaling 5 ml for each subject, were drawn into vacuum tubes. Immediately upon collection, samples were stored on ice. At the end of each daily collection, samples were centrifuged to separate serum and stored at -20 °C until analyses. Testosterone (T) and estradiol (E2) were measured by an automated chemo-iluminescence analyzer. During the process of determination, a parallel sample were measured every 7 samples. At the end of the measurement of all samples, 20 samples were randomly selected and retested. Kit was provided by Autobio Diagnostics Co. Ltd. (Zhengzhou, China).

Statistical Analysis

The database was established by using Epidata 3.0 software (Epidata 3.0 for windows, Epidata Association Odense, Denmark) with data double entered into the database by different people. All data were analyzed with SPSS 12.0 software. Kolmogorov-Smirnov test and Levene test were used to inspect the normality and homogeneity of variance of all data, showing that all data were in line with normality and homogeneity of variance. The distribution of the basic situation of children such as birth and feeding patterns, birth weight, maternal education, passive smoking status and so on was assessed by using the Chi-Square test. The levels of pollutants in river water samples were summarized with descriptive statistics. One-way analysis of variance (ANOVA) tests was used to compare mean differences in levels of pollutants of the three sections. The concentrations of pollutants in the drinking water and the indicators of physical development and sex hormone levels of children in the two areas were compared by using Independent-Sample T test. (significance level α= 0.05).

RESULTS

The Balance of Survey Subjects

Recovered valid questionnaires were analyzed, and the results showed that children in the two areas had no significant difference in the birth and feeding patterns, birth weight, maternal education, passive smoking, exercise time, sleep time etc. (P>0.05).

SY River Water Pollutant Levels

Compared with the standards of surface water quality[9], the concentrations of Pb and Hg exceeded Class V in each section and other indicators exceeded Class III. In addition to As, there were no significant differences in the concentrations of all concerned substances among the three sections (P>0.05). Compared with the US and DS, the concentrations of As in MS were significantly lower (P<0.05), but there was no significant difference between the US and DS (P>0.05) (Table 1).

Table 1. Concentrations of 8 Pollutants in SY River Water ($\bar{x}$ ±s)

Indicators/Section	US (n=9)	MS (n=9)	DS (n=9)	F	P
Pb (μg/L)	52.100±8.921	70.467±12.888	64.800±7.879	2.587	0.155
As (μg/L)	50.138±5.563	33.447±5.564*	48.283±8.499	5.615	0.042
Hg (μg/L)	2.244±0.140	2.282±0.279	2.402±0.145	0.521	0.619
DMP (μg/L)	69.107±7.663	73.951±14.902	75.975±10.712	0.283	0.763
DEP (μg/L)	81.103±18.503	80.406±6.812	82.240±15.219	0.012	0.988
DBP (μg/L)	11.613±5.641	11.603±1.745	10.203±1.715	0.157	0.858
DEHP (μg/L)	18.216±3.206	17.470±1.679	22.141±2.028	3.292	0.108
BPA (μg/L)	2.789±0.790	2.800±0.735	3.281±1.006	0.326	0.734

Drinking Water Pollutant Levels

Compared with the control area, the concentrations of Hg, Pb, As, BPA, DEP, and DBP in the drinking water of the polluted area were significantly higher (P<0.05); however, there were no significant differences in DMP and DEHP between the two areas (P>0.05) (Table 2).

Table 2. Concentrations of 8 Pollutants in Drinking Water ($\bar{X} \pm s$)

Indicators	Polluted Area (n=30)	Control Area (n=30)	t	P
Hg (μg/L)	0.752± 0.244	0.452±0.121	2.466	0.039
Pb (μg/L)	17.200± 8.311	7.200±1.202	2.663	0.029
As (μg/L)	31.025± 6.580	21.212±6.599	2.355	0.046
BPA (μg/L)	0.160± 0.113	0.003±0.002	3.116	0.036
DMP (μg/L)	38.189± 2.272	38.760±5.804	-0.205	0.843
DEP (μg/L)	44.044±25.644	13.269±9.940	2.502	0.037
DBP (μg/L)	0.931±0.236	0.542±0.286	2.342	0.047
DEHP (μg/L)	12.487±1.555	11.750±0.224	1.049	0.351

Anthropometric Indicators and Sex Hormones of Boys

The anthropometric indicators and sex hormones of boys are shown in Table 3. Values of the anthropometric indicators including height, weight, bust circumference and skinfold thickness among the boys of the polluted area were found to be higher than those in the control area, but there was no statistically significant difference (P>0.05). However, the E2 and T were significantly lower in the control area than in the polluted area (P<0.05). (Table 3).

Table 3. Values of Anthropometric Indicators and Sex Hormones of Boys in Two Areas ($\bar{X} \pm s$)

Indicators	Polluted Area (n=30)	Control Area (n=48)	t	P
Height (cm)	139.00±11.	137.48±7.3	0.651	0.518
Weight (kg)	34.00±6.19	31.44±6.25	1.705	0.093
Skinfold(cm)	1.77±0.82	1.42±0.57	2.011	0.050
Bust Bircumr-ence(cm)	66.12±5.38	66.11±5.47	0.008	0.994
E2 (pg/mL)	29.27±5.73	25.45±3.94	3.208	0.002
T (ng/mL)	0.33±0.19	0.23±0.14	2.491	0.016

Anthropometric Indicators and Sex Hormones of Girls

A total of 76 girls participated in this study. Although there were no significant differences between the polluted and control areas in height, weight and bust circumference (P>0.05), the skinfold thickness was significantly higher among the girls in the polluted area than in the control area (P<0.05). Compared with the control area, both the E2 and T were significantly higher in the polluted area (P<0.05). (Table 4).

Table 4. Values of Anthropometric Indicators and Sex Hormones of Girls in Two Areas ($\bar{X} \pm s$)

Indicators	Polluted Area (n=39)	Control Area (n=37)	t	P
Height(cm)	139.19±8.97	139.04±8.33	0.081	0.936
Weight(kg)	33.17±7.44	31.44±5.98	1.124	0.265
Skinfold thickness(cm)	1.99±0.77	1.56±0.51	3.036	0.003
Bust Circum-ference(cm)	66.12±7.69	65.68±5.52	0.289	0.774
E2(pg/mL)	31.52±5.54	27.14±4.36	3.798	0.001
T(ng/mL)	0.39±0.19	0.28±0.15	2.848	0.006

DISCUSSION

Drinking Water Influenced by SY River Pollution

Over the years, environmental protection departments at all levels have been growingly concerned about the Huai River pollution. Since beginning of management of the Huai River pollution in 1994, a decade's efforts have decreased the total emissions and improved the water quality to some extent[10]. However, recent years have also seen gradual increase of the Huai River pollution. According to the monitoring data, about 50% of the Huai River Basin exceeded Class V standards in 2004 in terms of the surface water quality, which could directly affect local people living around the Basin[1].

It could be known from this study that: 1) the contents of Pb and Hg exceeded Class V standards of surface water quality in each section and other indicators were more than III. 2) In addition to As, there were no significant differences in concentrations of concerned pollutants among the various sections. The above results suggest that the pollution of the SY River is still very serious and its self-purification capacity has disappeared.

In the current study, various pollutants, including heavy metals, polycyclic aromatic hydrocarbons and PAEs, were detected in the groundwater near the SY River at a depth of 20-50 m,

and even over 200 m[11], justifying the argument that according to the water level and changes of riverbed structure, the river water can enter into the ground and form groundwater by different ways[12-14]. The two main ways are as follows: one is the leaching-type which is the main way to supply groundwater in dry seasons, while the other is lateral seepage in rainy seasons. Because of the filtering action of soil, the concentrations of pollutants in groundwater were significantly lower than those in the river water, and a significant negative correlation was found between concentrations of pollutants in groundwater and the distance from the river[14]. In this study, concentrations of Pb, Hg, As, DEP, DBP, and BPA in the drinking water of the control area were significantly lower when compared to the polluted area, suggesting that the drinking water of polluted area might have been contaminated by the polluted river via horizontal spread, vertical infiltration, as well as rainwater dissolution.

Hormone Levels of Children Affected by Pollutants in Drinking Water

Previous studies have shown that drinking water pollution has a direct relationship with the damage of human health[5-8]. Children aged 8-13 years are in the second peak of growth and development. During this period, various systems are highly sensitive to environmental pollutants, especially the sexual development. And the hormone level is one of the important indicators of children sexual development. It could be learnt from this study that the serum concentrations of E2 and T were significantly higher in the polluted area than in the control area for both boys and girls. The difference in children's E2 and T levels between the two areas may be related to chemical pollution, which can interfere with composition and secretion of sex hormones. Other studies have revealed that Pb, Hg, As, PAEs, and BPA all have endocrine disrupting effects[15-20]. They can influence and regulate the sex hormone levels by hypothalamus-pituitary-gonad axis and thus affect growth and development of children[15]. Pb, Hg, As are highly toxic substances that can exist for a long time, and they are difficult to degrade in the environment and can affect the gonads of humans[18]. PAEs can affect the ovarian secretion function in mice, showing the proposed role of estrogen[17]. As an environmental endocrine disruptor, BPA also has dose-dependent estrogenic effects[19].

Influence of Polluted Water on Anthropometric

This research also showed that the children in the two areas had no significant difference in the birth and feeding patterns, birth weight, maternal education, exposure to passive smoking exercise time, sleep time and so on according to analyses of the valid questionnaires. Such finding suggested that there were no genetics, nutrition, exercise and other confounding factors to impact on the children's growth and development in the two areas, and therefore, the two areas had comparable physical development of children.

Skinfold thickness is an important indicator used to infer the body fat content and determine the development of subcutaneous fat. Since different people have distinct genetic quality, living environment, diet and so on, the body fat distribution and its percentage in the total body weight are likely to show their own characteristics. A study showed that children in the puberty had higher subcutaneous fat content because their body had secreted a lot of estrogen[21]. The result of this study indicated that the skinfold thickness of girls in the polluted area was significantly higher than that in the control area while the skinfold thickness of boys in the polluted area was higher than that in the control area but with no statistical significance. The reason may be that the children in the polluted area had a higher serum concentration of E2 as a result of the effects of EEDs. A high concentration of estrogen can make cells become larger and thus accumulate a lot of fat, resulting in a significant increase in skinfold thickness. As the puberty of boys was 1-2 years later than girls, the impact on girls may be larger than on boys. In view of this, the sample size shall be enlarged in future research. However, no significant difference was found among other indicators of physical growth. Findings of the present study are inconsistent with the results of another study[22], according to which physical development indexes of children living in the polluted area were significantly lower than those in the control area, with the types and concentrations of pollutants in the water not clearly indicated. One reason for such inconsistency may be that the types and concentrations of pollutants in the water under study may be different from those in other literature, which only caused a change in hormone levels, but did not lead to morphological abnormalities.

In summary, the results of the present study show that the pollution of the SY River is still very

serious, and has affected the groundwater quality. Meanwhile, the pollutants in the drinking water have interfered with composition and secretion of children's serum sex hormones to some extent. In consideration of the limitation of this study, it is essential to replicate these findings in different villages near the SY River with lager sample sizes as well as more detailed information on children development and growth.

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