Drug Latentiation

Synthesis and Preliminary Evaluation of Testosterone Derivatives

By S. MORRIS KUPCHAN*, ALAN F. CASY*†, and JOSEPH V. SWINTOSKY‡

Because of their potential increased *in vivo* activity following oral administration and their possible advantage over testosterone in regard to duration of action, a series of testosterone derivatives were prepared. It was assumed that these might be absorbed more efficiently or metabolized less readily as a result of the chemical modifications performed upon them, yet would be converted back to testosterone in the body by nonenzymatic or enzymatic processes. In the chemical phase of these studies, much of the initial work was performed with cholesterol as the model compound prior to attempting to make the testosterone derivatives. The syntheses of both the cholesterol and testosterone derivatives are reported. In addition to syntheses, preliminary androgenic and myotrophic evaluation tests of aqueous testosterone derivative suspensions, administered orally, were performed in rats. Tests of eight of the derivatives revealed that testosterone acid succinate (X), testosterone amidosuccinate (XIII*a*), and testosterone ethylene ketal methoxymethyl ether (XVI*b*) displayed anabolic and androgenic activity; testosterone pyruvate (II), testosterone ethyl succinate (XII), testosterone *N,N*-dimethylamidosuccinate (XIII*b*), and testosterone tetrahydropyranyl ether showed only androgenic activity. Testosterone glutarate (XI) showed a lower order of androgenic activity, and ditestosterone formal (XV*b*) was virtually inactive.

THIS PAPER describes the preparation of a number of steroidal compounds derived primarily from testosterone. The structural features of these derivatives, which suggest that they might undergo nonenzymatic or enzymatic cleavage to regenerate the parent compound, are discussed. The testing of the testosterone derivatives in castrated male rats for androgenic and myotrophic activities after oral administration as suspensions is reported also.

INTRODUCTORY DISCUSSION

Harper (1, 2) has used the term "latentiation" to describe the conversion of drugs to derivatives from which the parent drugs are regenerated upon *in vivo* enzymatic attack. The term "latentiation" is used here in a broader sense to include conversion of drugs to derivatives from which the parent drugs are regenerated *in vivo* by either enzymatic or nonenzymatic processes. This conception of drug latentiation enables chemically and biologically oriented scientists to unite forces at the level of molecular biology. Together, they can bring to bear on the problems of drug molecule design the rapidly unfolding knowledge which relates (*a*) chemical reactivity and kinetics to chemical structure in the absence of catalysts and (*b*) enzymatic reactivity to drug-substrate structure, enzyme and drug-substrate concentrations, and other factors influencing enzyme reactions.

Some differences in properties between parent drug and derivatives that may be reflected in differences in biological activities are as follows. (*a*) Differences in rates of absorption which may result, for example, from conversion of a polar to a less polar molecule, with a consequent increase in lipid solubility and thus a more favorable transport across lipoid barriers, such as the gastrointestinal tract and the blood-brain barrier (3). A decrease in polarity may result also in an increase in the storage of the derivative in body fat, leading in some instances to prolongation of activity. (*b*) Differences in rates of metabolism. If the functional group modified is one normally involved in the metabolism of the molecule, the rate at which the drug is eliminated from the body may be reduced—hence its action prolonged, *e.g.*, the masking of hydroxyl groups, the conjugation of which is a common metabolic pathway (4). (*c*) Differences in time of onset and duration of action. If derivative formation involves a functional group essential for activity, the modified drug may be ineffective until the original functional group has been regenerated; the period for onset of action may therefore be longer than it is for the original drug. Duration of effect may be correspondingly greater, since metabolic processes that reduce plasma concentration of the original drug may not operate so rapidly upon the modified form. (*d*) Differences in potency. Due to more favorable transport across lipid barriers and to resistance to metabolic processes, the derivative may concentrate at the target area in a higher concentration than that of the parent. Consequently, provided conversion to the active form occurs fairly rapidly at the site of action, enhanced potency of the derivative over the parent may result. If transformation is slow (other factors being equal or more favorable), lower potency would be expected but possibly coupled with increased duration of action. (*e*) Differences in rates of excretion. Excretion and metabolic processes are closely interrelated. If conversion of the substance to a more polar and thus more readily excretable form (*e.g.*, a conjugate) is not possible until the original functional group has been unmasked, elimination of the derivative from the body may be slower than that of the parent.

Received November 13, 1964, from the * Department of Pharmaceutical Chemistry, School of Pharmacy, University of Wisconsin, Madison, and ‡ Smith Kline and French Laboratories, Philadelphia, Pa.

Accepted for publication December 21, 1964.

The authors thank Dr. Harry Saunders, Smith Kline and French Laboratories, Philadelphia, Pa., for performing the bioassays in this study.

† Present address: Chelsea College of Science and Technology, London, England. This author thanks the Wellcome Trust for a travel grant.

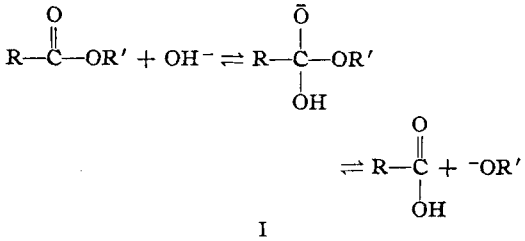
Testosterone was chosen as a model for these studies for the following reasons. (*a*) It has secondary alcohol and ketonic groups which enable the molecule to be converted readily to appropriate derivatives. (*b*) It has characteristic physiological effects for which reliable and well-established assay procedures have been devised. (*c*) There is a need for androgenic and myotrophic agents showing utility when administered orally and which might show extended duration of action. (*d*) The authors were interested in utilizing some adducts for steroid derivative synthesis which heretofore have not been utilized for drug latentiation. (*e*) It is well known that certain simple ester derivatives of testosterone, such as the propionate, cyclopentylpropionate, and phenylacetate, retain the qualitative physiologic effects of testosterone. They may be considered latentiated forms of testosterone which have a distinct utility in therapeutics. Reifenstein *et al.* (5), for example, report that the duration of action of these esters follows the order phenylacetate > cyclopentylpropionate > propionate when they are administered parenterally to humans.

In considering the preparation of testosterone compounds for oral administration, the possibility exists that derivatives might be made which are more active than testosterone itself. For example, this could result if testosterone, which is poorly soluble, is not completely absorbed, and if the derivatives were prepared with a view to facilitating their dissolution and absorption. Preparation of acidic derivatives capable of being solubilized in gastrointestinal fluids or preparation of derivatives having less symmetry than testosterone, lower melting points, and greater water or oil solubilities could facilitate the dissolution and absorption processes.

Alternatively, if the low potency of testosterone following oral administration results from metabolism in the liver, it is possible that temporarily masking a reactive group, such as hydroxyl, in testosterone might result in less destruction of potential testosterone activity during the passage of the derivatives through the liver following their absorption into the portal blood circulation.

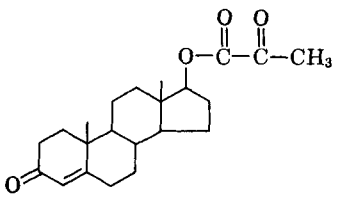
On the basis of their potential *in vivo* activity and their possible increased activity over testosterone itself, the investigation of the following derivatives of testosterone was proposed: (*a*) the pyruvate ester and the enol acetate pyruvate ester, (*b*) the acid succinate, glutarate, and derived substances, (*c*) the methoxymethyl ether, and (*d*) the dihydropyran adduct. Due to the high cost of testosterone, much of the initial chemical work was carried out with cholesterol as a model compound.

The anticipated facile *in vivo* conversion of such derivatives into the parent compound rests upon both *in vitro* and *in vivo* chemical evidence. For instance, esters of acids possessing electron withdrawing groups are known to be subject to facile *in vitro* hyrolysis. Bimolecular base catalyzed hydrolysis of esters is believed to proceed (6) through a tetrahedral addition intermediate (I). This intermediate is negatively charged, and it is to be anticipated that electron-releasing substituents in the acid moiety would retard and electron-withdrawing substituents accelerate hydrolysis of esters by this mechanism. Quantitative studies of the effects of substituents of differing electronic properties upon second-order alkaline hydrolysis rates of

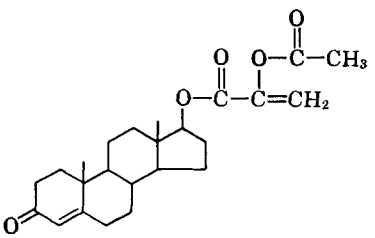


I

esters yielded results in full support of these expectations (6, 7). The rate of alkaline hydrolysis of ethyl pyruvate was found to be particularly high. Furthermore, Sudborough (8) found ethyl pyruvate to undergo substantial hydrolysis, even in water. The synthesis of testosterone pyruvate (II) was therefore proposed, since its ready *in vivo* hydrolysis is a reasonable expectation. The acid moiety, pyruvic acid, is a normal physiological acid, and its liberation should not lead to undesirable side effects. As an extension, the preparation of pyruvate derivatives, such as the enol acetate (III), was considered.

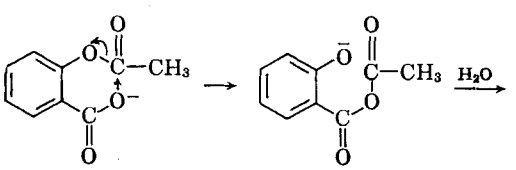


II

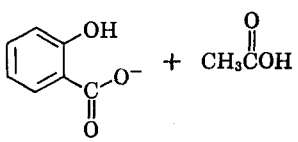


III

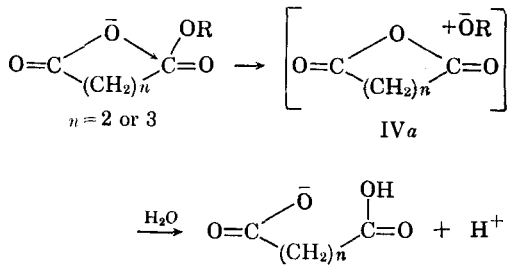
Hydrolysis of esters of dicarboxylic acids is facilitated by intramolecular catalysis by carboxylate ion. This mechanism of ester hydrolysis facilitation was first advanced (9) to account for the hydrolysis of aspirin in the pH range 4 to 8. Aspirin hydrolysis is catalyzed by hydrogen ions below this range and by hydroxide ions above it, but within the range the rate is independent of pH. The



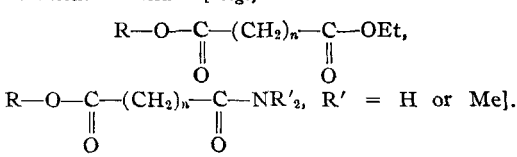
IV



mechanism is considered to involve attack by carboxylate ion upon the ester carbonyl carbon atom, forming an anhydride intermediate (IV) that is broken down rapidly by water. Morawetz *et al.* (10, 11) measured the rates of hydrolysis of a series of phenyl acid glutarates and acid succinates. These esters were hydrolyzed at an unusually fast rate in the intermediate pH region of 3 to 8. Rapid solvolysis rates were accounted for by a mechanism similar to that shown above for aspirin, involving substituted glutaric and succinic anhydrides as intermediates (IV*a*). Attempts to prepare the mono-



choline ester of succinic acid by half-hydrolysis of succinyl choline gave the starting material and succinic acid (12). Bender (9) quotes this fact as evidence for the facile hydrolysis of half-esters of succinic acid. Because of the evidence of assisted hydrolysis at neutral and near neutral pH values in hemiesters of glutaric and succinic acids, it was proposed to prepare these and related esters of testosterone. Testosterone derivatives considered were esters with lower aliphatic alcohols, and primary and substituted amides [*e.g.*,



Conversion of alcohols to lower alkyl *ethers* produces derivatives relatively stable to the action of both acids and alkalies and that generally require vigorous conditions to effect their cleavage (*e.g.* heating with concentrated hydrogen bromide or hydrogen iodide). This stability also holds under conditions that prevail in the body. Simple ethers are excreted unchanged; in only a few cases has the *in vivo* cleavage of an aliphatic ether been demonstrated, although aromatic methyl ethers are demethylated fairly readily (4). The ether chosen for this present study—namely, the methoxymethyl ether and the dihydropyran adduct of testosterone—differ from ethers discussed above because they are easily cleaved by treatment with dilute acid (13–16). The reason for their unusual instability is that both ethers are strictly acetals; such aldehyde derivatives are unchanged by alkali but decomposed by acid. Thus, methoxymethyl ether, R—O—CH_2—O—CH_3, is an acetal derived from R—OH, methanol, and formaldehyde; a dihydropyran adduct is a cyclic acetal derived from R—OH and 5-hydroxypentanal.

Though the preceding comments emphasize the possibilities for base or acid catalyzed hydrolytic cleavage, it is recognized that enzyme-mediated cleavage of these compounds *in vivo* also is highly probable. Body enzymes possess configurations which may complement the geometry and polarization of certain more simple molecules or functional groups. The nature of some enzyme-mediated reactions has been discussed in recent literature (17–19, 45). If the substrate and the enzyme at some sites possess a degree of complementariness to facilitate their proper orientation with regard to each other, this is a necessary prelude to modification of the substrate; but this does not assure it. Also required is a complementariness between the substrate functional group to be acted upon and the functional operator of the enzyme molecule. This operator may be an electron donor or other suitably placed group which can carry out its catalytic function efficiently in this intramolecular environment because it is within range of the contiguous receptive group of the substrate. When a reaction is mediated through enzymes within the body, the activation energy may be reduced appreciably, and the speed of the reaction may be far in excess of that of the uncatalyzed reaction. It is reasonable to assume that some of the testosterone derivatives described here may exhibit a degree of susceptibility to enzymatic cleavage processes.

EXPERIMENTAL AND RESULTS

Melting points, determined on a Fisher-Johns hot stage, are corrected. Infrared spectra were measured in solutions in chloroform on a Beckman model IR5 spectrophotometer with NaCl prism and plates, using 0.1-mm. NaCl cells. All adsorption chromatography, unless otherwise specified, was carried out on Merck alumina. Skelly B refers to petroleum ether, b.p. 60–80°. Microanalyses were carried out by Dr. S. M. Nagy and associates, Massachusetts Institute of Technology, Boston.

Commercial pyruvic acid (Eastman's White Label) gave a colorless mobile distillate (b.p. 70–72°/22 mm.) and left a considerable quantity of residue that solidified on cooling. Redistilled material suffered little change after storage for several months in a refrigerator.

Cholesteryl Pyruvate.—A mixture of cholesterol (15.4 Gm., 0.04 mole), redistilled pyruvic acid (21.1 Gm., 0.24 mole), and *p*-toluenesulfonic acid (200 mg.) in dry benzene (200 ml.) was heated for 5 hr. under reflux in a flask to which a Dean-Stark water take-off tube was attached. The benzene was evaporated under reduced pressure and the residue shaken with Skelly B (200 ml.). The mixture was filtered and the filtrate concentrated, whereupon cholesteryl pyruvate (12.0 Gm.), m.p. 107.5–108.5°, separated. An analytical sample, recrystallized from Skelly B, had m.p. 108.5–109.5°; $[\alpha]_D^{30}$ −40.5° (c, 1.8 in $CHCl_3$).

Anal.—Calcd. for $C_{30}H_{48}O_3$: C, 78.9; H, 10.5. Found: C, 78.8; H, 10.4.

The infrared spectrum showed a strong absorption peak at 5.82 μ with a small shoulder at 5.78 μ.

Testosterone Pyruvate (II).—A mixture of testosterone (5 Gm., 0.017 mole), redistilled pyruvic acid (9.17 Gm., 0.104 mole), and *p*-toluenesulfonic acid (87 mg.) in benzene (75 ml.) was heated under reflux for 5 hr. as described under *Cholesteryl Pyruvate*. The benzene was evaporated under reduced pressure and the residue extracted several times with hot Skelly B. The extracts were

filtered and concentrated and the residue (5.45 Gm.) recrystallized from ethyl acetate to give testosterone pyruvate (2.65 Gm.), m.p. 122–123°. A further crop (1.11 Gm.), m.p. 119.5–120.5°, separated from the mother liquor.

Anal.—Calcd. for $C_{22}H_{30}O_4$: C, 73.7; H, 8.4. Found: C, 73.55; H, 8.2.

The infrared spectrum showed strong absorption peaks at 5.82 μ (sh at 5.78 μ) and 6.05 μ; $[\alpha]_D^{29}$ +106° (c, 1.9 in $CHCl_3$).

2:4-Dinitrophenylhydrazone of Cholesteryl Pyruvate.—2:4-Dinitrophenylhydrazine (19.8 Gm., 0.1 mole) was suspended in a solution of distilled pyruvic acid (9.7 Gm., 0.11 mole) in water, and the mixture was stirred and heated to 70–80° for 1 hr. The solid was collected, washed, and recrystallized from glacial acetic acid (300 ml.) to give the 2:4-dinitrophenylhydrazone of pyruvic acid (16.5 Gm.), m.p. 214.5–215.5°. [Lit. (20) m.p. 216°.] The latter derivative (13.8 Gm.), thionyl chloride (30 ml.), and xylene (60 ml.) were heated under reflux for 5 hr. The 2:4-dinitrophenylhydrazone of pyruvoyl chloride (6.7 Gm.), m.p. 178–179° [lit. (20) m.p. 173–176° dec.], separated from the mixture on cooling. A mixture of this acid chloride (3.14 Gm., 0.11 mole), cholesterol (3.86 Gm., 0.1 mole), pyridine (1 Gm., 0.12 mole), and toluene (100 ml.), heated for 1 hr. under reflux, gave on cooling the 2:4-dinitrophenylhydrazone of cholesteryl pyruvate (5.5 Gm.), m.p. 280° dec. An analytical sample, recrystallized from toluene, had m.p. 282–283° dec.

Anal.—Calcd. for $C_{36}H_{52}N_4O_6$: C, 67.9; H, 8.2; N, 8.8. Found: C, 67.9; H, 8.2; N, 8.8.

Enol Acetate of Cholesteryl Pyruvate (III).—A solution of cholesteryl pyruvate (2.74 Gm.) and *p*-toluenesulfonic acid (1.03 Gm.) in acetic anhydride (225 ml.) was distilled slowly over 4.5 hr. The residual acetic anhydride (approximately 50 ml.) was evaporated under reduced pressure, the residue dissolved in ether, and shaken with water to which aqueous sodium hydroxide was added gradually until the reaction of the water was neutral. The organic phase was dried, the ether evaporated, and the residue crystallized several times from Skelly B to give the enol acetate of cholesteryl pyruvate (0.2 Gm.), m.p. 135°.

Anal.—Calcd. for $C_{32}H_{50}O_4$: C, 77.1; H, 10.0. Found: C, 76.9; H, 9.6.

The infrared spectrum showed absorption peaks at 5.8, 6.2, and 8.0 μ; $\lambda_{max.}^{alc.}$ 240 mμ, (ϵ 5900).

Action of Pyruvic Acid on Testosterone Dioxolane.—A mixture of testosterone dioxolane (3.32 Gm., 0.01 mole), redistilled pyruvic acid (5.28 Gm., 0.06 mole), and *p*-toluenesulfonic acid (50 mg.) in dry benzene (60 ml.) was heated under reflux (Dean-Stark attachment) for 5 hr. The benzene was evaporated under reduced pressure and the residue extracted several times with cold Skelly B. The extracts were filtered, concentrated, and the residue (3.56 Gm.) crystallized from ethyl acetate to give testosterone pyruvate (1.22 Gm.), m.p. and mixed m.p. 119.5–121°.

3-Methoxy-$\Delta^{3,5}$-androstadien-17β-ol Pyruvate (IX).—A mixture of testosterone pyruvate (1 Gm.), *p*-toluenesulfonic acid (26 mg.), methanol (0.2 ml.), 2,2-dimethoxypropane (5 ml.), and dimethylformamide (5 ml.) was heated (oil-bath) under reflux for 3.5 hr. The cooled product was diluted with ether and shaken with water containing sodium bicarbonate (12 mg., equivalent to the *p*-toluenesulfonic acid). The organic phase was washed with water, dried (Na_2SO_4), and the solvents evaporated. The residue (1.16 Gm.) was crystallized from ethyl acetate to give 3-methoxy-$\Delta^{3,5}$-androstadien-17β-ol pyruvate (0.5 Gm.), m.p. 143–145°. An analytical sample crystallized from ethyl acetate had m.p. 146–149°; $[\alpha]_D^{28}$ −131° (c, 1.8 in $CHCl_3$).

Anal.—Calcd. for $C_{23}H_{32}O_4$: C, 74.2; H, 8.6. Found: C, 74.2; H, 8.6.

The infrared spectrum showed absorption peaks at 6.02 and 6.12 μ (enol ether doublet); $\lambda_{max.}^{alc.}$ 240 mμ, (ϵ 19800).

Testosterone Acid Succinate (X).—A mixture of testosterone (2.5 Gm.), succinic anhydride (2.5 Gm.), and pyridine (12.5 ml.) was heated under reflux for 4.5 hr. The cooled product was diluted with ether and extracted with aqueous sodium hydroxide until the aqueous phase was alkaline. The latter was acidified with hydrochloric acid and cooled in ice water. The solid which separated was collected, dried, and crystallized from benzene to give testosterone acid succinate (2.86 Gm.), m.p. 186–187°. [Lit. (21), m.p. 183–185° and 191–193°.]

Testosterone Acid Glutarate (XI).—Compound XI, m.p. 168.5–169°, was prepared in a manner similar to the succinate, from glutaric anhydride.

Anal.—Calcd. for $C_{24}H_{34}D_5$: C, 71.65; H, 8.5. Found: C, 71.5; H, 8.5.

Ethyl Ester of Testosterone Acid Succinate (XII).—Oxalyl chloride (0.32 Gm., 0.0025 mole) in dry benzene was added to a solution of testosterone acid succinate (1 Gm., 0.0025 mole) in the same solvent and the mixture heated for 1.5 hr. in an oil-bath kept between 70–90°. The benzene was evaporated under reduced pressure and absolute alcohol (10 ml.) added to the residue. The alcohol was evaporated, the residue dissolved in ether and shaken with saturated aqueous sodium bicarbonate, then water. After drying (Na_2SO_4), the ether was evaporated, and the residue (1.05 Gm.) in benzene was chromatographed on alumina. The benzene eluate yielded crude material (0.5 Gm.) that was crystallized from isopropyl ether to give the ethyl ester of testosterone acid succinate (0.25 Gm.), m.p. 98°; $[\alpha]_D^{29}$ +83.3° (c, 2.1 in $CHCl_3$).

Anal.—Calcd. for $C_{25}H_{36}O_5$: C, 72.1; H, 8.65. Found: C, 72.5; H, 8.9.

Further elution of the column, using 1% methanol in benzene, gave material (0.19 Gm.) that did not depress the melting point of and had an infrared spectrum identical to that of testosterone.

Amide of Testosterone Acid Succinate (XIIIa).—Dry ammonia gas was passed for 10 min. into a solution of crude acid chloride in benzene derived from testosterone acid succinate (1.5 Gm.) and oxalyl chloride (0.49 Gm.), as described above. The product was dissolved in chloroform-ether, shaken with saturated aqueous sodium bicarbonate, then with water. After drying (Na_2SO_4), the solvents were evaporated; the residue (1.6 Gm.), which solidified on being stirred with benzene, was crystallized from ethyl acetate to give the amide of testosterone acid succinate (0.5 Gm.), m.p. 156–157°; $[\alpha]_D^{28}$ +79.5° (c, 2.5 in $CHCl_3$).

Anal.—Calcd. for $C_{23}H_{33}NO_4$: C, 71.3; H, 8.5; N, 3.6. Found: C, 70.5; H, 8.6; N, 3.5.

The infrared spectrum showed absorption peaks at 2.85 and 2.9 μ (free and bonded N—H), 5.8 μ (ester and primary amide carbonyl), and 6.0 and 6.2 μ (Δ^4-3-keto system).

N,N-Dimethylamide of Testosterone Acid Succinate (XIIIb).—Dry dimethylamine gas was passed for 0.5 hr. into a solution in benzene of crude acid chloride, derived from testosterone acid succinate (2 Gm.) and oxalyl chloride (0.64 Gm.). Alkali-soluble material was removed, as described above for the primary amide, and the residue (2 Gm.) in benzene chromatographed on alumina. The benzene eluate yielded crude tertiary amide (0.75 Gm.) which was crystallized from isopropyl ether–methanol to give the *N,N*-dimethylamide of testosterone acid succinate (0.35 Gm.), m.p. 141–142°, $[\alpha]_D^{27}$ +70.5° (c, 2.7 in $CHCl_3$).

Anal.—Calcd. for $C_{25}H_{37}NO_4$: C, 72.3, H, 8.9; N, 3.4. Found: C, 72.4; H, 8.9; N, 3.4.

Testosterone (0.9 Gm.) was obtained from the 1% methanol in benzene eluate.

N,N-Dimethylamide of Cholesteryl Acid Succinate.—Cholesteryl acid succinate (3.18 Gm., 0.0065 mole), m.p. 181–182° [lit. (22) m.p. 175°], prepared from cholesterol and succinic anhydride, and oxalyl chloride (1.7 Gm., 0.013 mole) were dissolved in dry benzene (50 ml.) and the solution heated under reflux for 3 hr. Volatile material was evaporated under reduced pressure, the residue dissolved in benzene, re-evaporated, and heated under reflux for 4 hr. with dimethylformamide (10 ml.). The solid which separated on cooling was recrystallized from dimethylformamide to give the *N,N*-dimethylamide of cholesteryl acid succinate (2 Gm.), m.p. 121.5–122.5°. An analytical sample recrystallized from dimethylformamide had m.p. 123.5–124.5°.

Anal.—Calcd. for $C_{33}H_{55}NO_3$: C, 77.2; H, 10.7; N, 2.7. Found: C, 76.7; H, 10.4; N, 2.9.

The infrared spectrum showed absorption peaks at 5.8 μ (ester carbonyl) and 6.1 μ (*tert*-amide carbonyl).

Testosterone-Dihydropyran Adduct (XIV).—Testosterone (1.44 Gm., 0.005 mole) was suspended in dry ether (65 ml.) and redistilled dihydropyran (1.26 Gm., 0.015 mole), and 3 drops of a solution of *p*-toluenesulfonic acid (0.5 Gm.) in ether (50 ml.) was added. The mixture was stored in the dark and shaken occasionally. Every 3 days for 3 wks., 10% dihydropyran in ether (4.5 ml.) and 3 drops of the acid catalyst solution were added. Solution was complete at the end of 1 wk. After 3 wk., the solution was shaken with saturated aqueous sodium bicarbonate, then with water. After drying (K_2CO_3), the solution was evaporated; the residue slowly solidified after storage in a vacuum desiccator and was crystallized several times from isopropyl ether to give the adduct (0.43 Gm.), m.p. 104.5–106.5°. [Lit. (23) m.p. 98–100°.]

Dicholesteryl Formal (XVa).—Cholesterol (3.12 Gm., 0.008 mole) was added to a suspension of sodium hydride dispersion (Metal Hydrides, Inc.) (0.24 Gm., 0.01 mole NaH) in dry benzene (50 ml.). The mixture was refluxed for 0.5 hr., cooled, treated with chloromethyl methyl ether (1.1 Gm., 0.013 mole), shaken, and left overnight. Water was added carefully, the organic phase separated, dried (Na_2SO_4), and evaporated. The residue was crystallized from ethyl acetate to give dicholesteryl formal (0.63 Gm.), m.p. 186.5–187.5°. An analytical sample recrystallized from ethyl acetate–chloroform had m.p. 190.5°; $[\alpha]_D^{29}$ −30.8° (c, 2.3 in $CHCl_3$).

Anal.—Calcd. for $C_{55}H_{92}O_2$: C, 84.2; H, 11.7. Found: C, 83.9; H, 11.5.

The infrared spectrum showed no absorption peaks in the O—H region and a strong peak at 8.64 μ (ether C—O). Mol. wt. (Rast) required for $C_{55}H_{92}O_2$: 784. Found: 675.

Concentrated sulfuric acid was added dropwise to a mixture of cholesterol (2 Gm.), ether (30 ml.), and formalin (50 ml.), and the product was shaken well and left for 3 days. The organic phase was separated, washed with water, dried (Na_2SO_4), and evaporated. The residue in Skelly B was chromatographed on alumina. Material was obtained in low yield (0.15 Gm.) from the Skelly B eluate which, after crystallization from ethyl acetate, did not depress the melting point of and showed an infrared spectrum identical to the dicholesteryl formal obtained above.

3-Methoxymethyl 5-Cholestene (XVIa).—Sodium strip (0.25 Gm., 0.011 atoms) was cut into a solution of naphthalene (1.28 Gm., 0.01 mole) in dry 1,2-dimethoxyethane (20 ml.) and the mixture stirred under dry nitrogen. A dark green color developed after a few minutes, and the temperature of the mixture remained between 22 and 25° during the stirring period. After 2.5 hr., cholesterol (3.5 Gm., 0.009 mole) in 1,2-dimethoxyethane (35 ml.) was added dropwise. A white solid precipitated, and the color of the mixture changed from dark green to pale grey. When addition was complete, the mixture was stirred for another 0.5 hr., then treated with freshly distilled chloromethyl methyl ether (0.8 Gm., 0.01 mole) in a few milliliters of 1,2-dimethoxyethane. The product was stirred for several hours, diluted with ether, then shaken with saturated aqueous sodium bicarbonate, followed by water. After drying (K_2CO_3), the solvents were evaporated and the residue (5 Gm.) chromatographed in Skelly B on alumina (100 Gm.). Naphthalene (1 Gm.) was recovered from Skelly B eluates. Elution with 25% benzene in Skelly B gave a solid (2.84 Gm.) which was crystallized from acetone to give 3-methoxymethyl 5-cholestene, m.p. 85.5–86.5°.

Anal.—Calcd. for $C_{29}H_{50}O_2$: C, 80.9, H, 11.6. Found: C, 80.8; H, 11.7.

The infrared spectrum showed a strong absorption peak at 8.7 μ (ether C—O).

Ditestosteryl Formal (XVb).—A few drops of water were added to a solution of testosterone (2 Gm., 0.007 mole) and chloromethyl methyl ether (1.12 Gm., 0.014 mole) in benzene (50 ml.) and the mixture shaken occasionally for 3 days at room temperature. The product was shaken with aqueous sodium bicarbonate and the aqueous phase dried (K_2CO_3). The solvent was evaporated and the residue recrystallized twice from chloroform–ethyl acetate to give ditestosteryl formal (0.47 Gm.), m.p. 223–225°. A further crystallization gave an analytical sample (0.28 Gm.), needles, m.p. 227–229°; $[\alpha]_D^{27}$ +121.2° (c, 2.0 in $CHCl_3$).

Anal.—Calcd. for $C_{39}H_{56}O_4$: C, 79.6; H, 9.5. Found: C, 79.5; H, 9.5.

The infrared spectrum showed a strong absorption peak at 8.6 μ (ether C—O). Mol. wt. (Rast) required for $C_{39}H_{56}O_4$: 588. Found: 491.

17 - Methoxymethyl - testosterone Dioxolane (XVIb).—Testosterone dioxolane (2.3 Gm.) was treated, as described above, with sodium naphthyl prepared from sodium (0.18 Gm.) and naphthalene (0.92 Gm.) in 1,2-dimethoxyethane and chloromethyl methyl ether (0.6 Gm.) in the same solvent added to the mixture. The product was processed and chromatographed, as described above for the cholesterol analog. Elution with 50% benzene in Skelly B gave a solid (1.4 Gm.) which was crystallized from isopropyl ether to give 17-methoxymethyltestosterone dioxolane, m.p. 135–136°; $[\alpha]_D^{28}$ −47.2° (c, 1.6 in $CHCl_3$).

Anal.—Calcd. for $C_{23}H_{36}O_4$: C, 73.4; H, 9.6. Found: C, 73.5; H, 9.5.

Testosterone Dioxolane (XVII) and Isomeric Product (XIX).—A mixture of testosterone (2.88 Gm., 0.01 mole), ethylene glycol (1.2 ml., 0.02 mole), and *p*-toluenesulfonic acid (45 mg.) in dry benzene (50 ml.) was heated under reflux for 6 hr. in a flask to which a Dean-Stark tube was attached. The solution was shaken with saturated aqueous sodium bicarbonate, then with water. After drying (Na_2SO_4), the benzene was evaporated and the solid residue crystallized from methanol–water to give testosterone dioxolane (1.26 Gm.), m.p. 185–186° [Lit. (24) m.p. 185–188°]; $[\alpha]_D^{28}$ −63.0° (c, 1.5 in $CHCl_3$).

When the above reaction was repeated using testosterone (3 Gm., 0.0104 mole), ethylene glycol (13.5 ml., 0.26 mole), and *p*-toluenesulfonic acid (45 mg.), the solid residue (3.4 Gm.) obtained was crystallized from a mixture of isopropyl ether (45 ml.) and pyridine (9 ml.) to give crystals (0.52 Gm.), m.p. 200–208°. One further crystallization from the same solvent mixture gave crystals (0.34 Gm.), m.p. 215–218°. An analytical sample had m.p. 221–223°; $[\alpha]_D^{27}$ + 101.6° (c, 1.8 in $CHCl_3$).

Anal.—Calcd. for $C_{21}H_{32}O_3$: C, 75.9; H, 9.6. Found: C, 75.7; H, 9.45.

The infrared spectrum showed a weak absorption band at 6.02 μ.

Acetic anhydride (0.5 ml.) was added to a warm solution of the isomeric product (0.25 Gm.) in pyridine (2.66 ml.). The next day the mixture was evaporated and the residue crystallized from isopropyl ether to give the acetylated isomer (0.12 Gm.), m.p. 160–161°; $[\alpha]_D^{28}$ +66.6° (c, 2.2 in $CHCl_3$). A further crop (0.18 Gm.), m.p. 159–160°, was obtained from the mother liquors.

Anal.—Calcd. for $C_{23}H_{34}O_4$: C, 73.3; H, 9.1. Found: C, 73.15; H, 9.2. It had absorption peaks at 5.8 μ (ester C=O) and 6.02 μ (C=C).

The NMR spectrum[1] of the Δ^4-dioxolane acetate in deuteriochloroform, examined after storage at room temperature for several weeks, exhibited the following characteristic signals: 4.35τ (C-4 proton of Δ^4-isomer), 4.73 (multiplet, C-6 proton of Δ^5-isomer), 5.3τ (multiplet, C-17 proton of both isomers), 6.12 and 6.35τ (dioxolane bimethylene groups of Δ^4- and Δ^5-isomers), 7.98τ (C-17 acetyl of both isomers), 8.82 and 9.00τ (19-methyl of Δ^4- and Δ^5-isomers), 9.18 and 9.22τ (18-methyl of Δ^4- and Δ^5-isomers). The integral showed the isomers to be present in approximately equal amounts. The NMR spectrum of the Δ^5-dioxolane acetate in deuteriochloroform, stored for the same period, was almost identical to the above.

[1] Measured on a Varian A-60 spectrometer with tetramethylsilane as internal standard.

Regeneration of Testosterone from XIX.—A solution of XIX (170 mg.) and *p*-toluenesulfonic acid (8.5 mg.) in dry acetone (8.5 ml.) was heated under reflux for 16 hr. The solvent was removed and the residue crystallized from acetone–isopropyl ether to give rosettes (50 mg.), m.p. 150–151°, that did not depress the melting point of testosterone. The infrared spectra of the respective samples were superimposable.

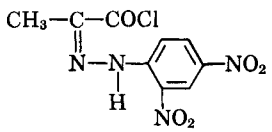
Test for Androgenic and Myotrophic Activities.—The oral androgenic-myotrophic activities of the various esters and related derivatives of testosterone were determined by a modification of the method of Eisenberg and Gordan (25). Young male rats of the Charles River strain, weighing 50 to 60 Gm., were castrated 3 to 7 days after receipt. Approximately 30 days later, they were divided into groups, with a minimum of six animals per group. The steroids were suspended in 0.5% aqueous tragacanth and administered orally (intubation) at a dose of 25 mg./Kg. body weight/day for 7 days. On the eighth day, the animals were sacrificed by decapitation, and the seminal vesicles and levator ani muscles were removed and weighed. The results were analyzed statistically by the Student *t* test for the significance of the increases in the weights of the seminal vesicles and levator ani muscles with respect to the castrate controls. For easy comparison, the results are presented in Table I as mean per cent increase over the castrate control values. Testosterone and methyltestosterone were employed as reference standards.

DISCUSSION

The normal procedure for the esterification of sterols is treatment with an acid chloride in the presence of base; this procedure was not considered in this work because of reported difficulties in preparing pyruvoyl chloride (26) and to the lability of pyruvate esters to alkali. Pyruvates of several alcohols have been prepared by heating an alcohol with excess of pyruvic acid, with or without an acid catalyst; in some cases, azeotropic removal of water was employed (27–29). The present syntheses were modeled on these reports. A solution of cholesterol (1 mole) and pyruvic acid (6 moles) in benzene, with a small amount of *p*-toluenesulfonic acid as catalyst, was heated under reflux; a Dean-Stark collecting tube was attached to the flask. Water collected in the side arm, but this could not serve as a criterion of reaction since the freshly distilled pyruvic acid used contained water. The course of the reaction was followed by means of thin-layer chromatography on hardened silica gel. Cholesterol and samples of the reaction mixture taken periodically were spotted onto the starting line of a prepared plate, which was developed for about 1 hr. in a tank containing 1% methanol in chloroform. The plate was air-dried, sprayed with 50% sulfuric acid in water, and dried at 100° until the spots were visible (steroids developed a purple color). A spot faster moving than that of cholesterol soon was apparent. Its ester nature was indicated by the fact that it moved at approximately the same speed as a spot due to cholesteryl acetate. Streaking made it difficult to decide whether the reaction had gone to completion, and the reaction was arbitrarily stopped after 5 hr. Excess of pyruvic acid was separated from steroid material by removing the benzene and

extracting the residue with petroleum ether, in which pyruvic acid is insoluble. Cholesteryl pyruvate separated in good yield from the concentrated petroleum ether extract. Its infrared spectrum showed an intense peak at 5.82 μ; absorption in the 3 μ region (OH) was negligible. Attempted chromotographic separation on acid-washed alumina and on Florisil gave cholestrol as the major product. These results indicate the lability of the pyruvate ester. Reaction of testosterone with pyruvic acid under the same conditions gave testosterone pyruvate in satisfactory yield; its infrared spectrum in the 5.8 μ region was similar to that of cholesteryl pyruvate.

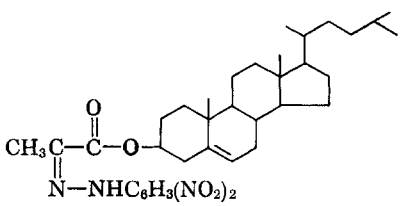
In contrast to pyruvoyl chloride, the acid chloride (V) is a well-defined stable compound (20), and its



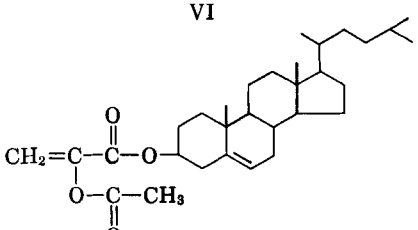
V

use in preparing pyruvates was investigated at an early stage in this work. Treatment of cholesterol with a mixture of the acid chloride (V) and pyridine gave the pyruvoyl derivative (VI) in high yield. However, attempts to regenerate the carbonyl group by heating with excess of cyclohexanone or of pyruvic acid were unsuccessful.

Treatment of cholesteryl pyruvate with isopropenyl acetate, using *p*-toluenesulfonic or sulfuric acid as catalyst (30), failed to give the corresponding enol acetate (VII). Slow distillation of a solution of the pyruvate in acetic anhydride (*p*-toluenesulfonic acid catalyst) gave the enol acetate (VII)



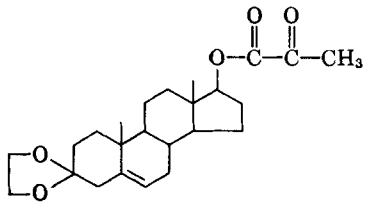
VI



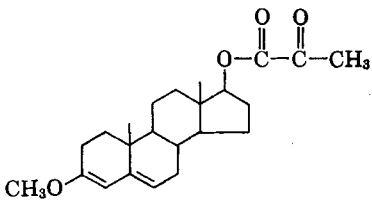
VII

in low yield (31). The product crystallized poorly and attempted chromatographic purification on alumina gave cholesterol as the major product. Its infrared spectrum was consistent with its formulation as an enol acetate, with absorption peaks at 5.8 μ (C=O), 6.18 μ (conjugated C=C), and 8 μ (acetate). The ultraviolet spectrum showed an absorption maximum at 240 mμ (ϵ 5940) in ethanol, further supporting the O=C—C=C structure; cholesteryl pyruvate showed only end absorption.

Application of this reaction to the pyruvate of testosterone dioxolane (VIII) rather than to testos-



VIII



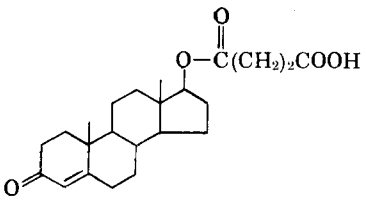
IX

terone was planned, since in the former compound the Δ^4-3-keto system is blocked and the possibility of its simultaneous conversion to an enol acetate excluded. An attempt to prepare testosterone pyruvate dioxolane from testosterone dioxolane and pyruvic acid by the described procedure unmasked the Δ^4-3-keto group and gave testosterone pyruvate. Although pyruvic acid has been used widely to regenerate steroid carbonyl compounds from derivatives, such as semicarbazones and phenylhydrazones (32, 33), its action upon dioxolanes apparently has not been noted in the literature. No crystalline product could be isolated after reaction of testosterone pyruvate with acetic anhydride.

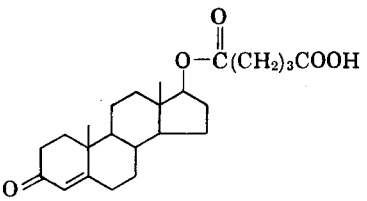
Ercoli and Gardi (34) found that enol ethers of Δ^4-3-keto steroids showed enhanced hormonal activity after oral administration but decreased activity by parenteral administration relative to the parent hormones. In view of this somewhat paradoxical result, it was considered of interest to prepare an enol ether of testosterone pyruvate. Enol ethers conventionally are prepared by acid-catalyzed treatment of ketones with orthoformic esters (35). However, Nussbaum *et al.* (36) noted that treatment of Δ^4-3-ketosteroids with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid gave corresponding methyl enol ethers. Furthermore, this reaction was specific for the ring A enone site. Reaction between testosterone pyruvate and 2,2-dimethoxypropane, as described, gave a crystalline product, formulated as the 3-methyl enol ether (IX). The infrared spectrum lacked the characteristic Δ^4-ketone bands but showed instead a doublet (6.02 μ and 6.12 μ) observed for $\Delta^{3,5}$-3-enol ethers (37). Its ultraviolet spectrum ($\lambda_{max.}$ 240 mμ, ϵ 19830) was also typical of such compounds (36).

Cholesterol acid succinate and testosterone acid succinate (X) have both been reported (21, 22) and were prepared in this work by heating a solution of the steroid and succinic anhydride in pyridine. Testosterone acid glutarate (XI) was prepared in a similar manner using glutaric anhydride. The preparation of derivatives of these hemiesters required their initial conversion to acid chlorides. Since Hancock and Linstead (38) recommended the

use of oxalyl chloride for the conversion of half-esters of succinic, glutaric, and phthalic acid to acid chlorides, this reagent was employed in the present work. Testosterone acid succinate was treated with 1 mole of oxalyl chloride in dry benzene, the volatile material distilled under reduced pressure, and dry ethanol added to the residue. After the removal of alkali-soluble materials, the oily product in benzene was chromatographed on alumina. Initial fractions contained the desired ethyl ester (XII) in moderate

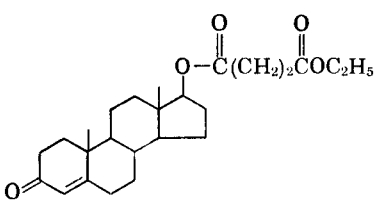


X

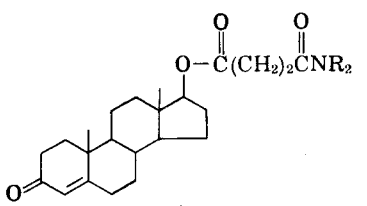


XI

yield. Elution with 1% methanol in benzene gave testosterone, showing that cleavage of the succinyl-sterol ester linkage occurs as a side reaction. In a subsequent experiment, with seed material available, the crude ester was crystallized directly.



XII

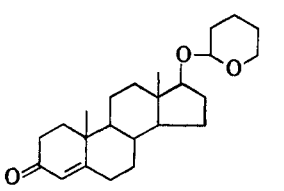


XIII

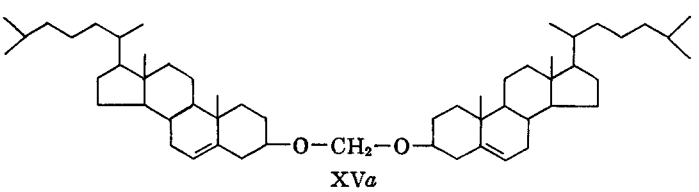
The primary amide (XIII*a*, R = H) was obtained in good yield by passing dry ammonia gas into the crude acid chloride, prepared as previously. Its infrared spectrum showed peaks at 2.85 and 2.9 μ (free and bonded N—H), 5.8 μ (C=O, ester and primary amide), and 6.0 and 6.2 μ (Δ^4-3-keto-system). Reaction of the acid chloride with dimethylamine gas gave the tertiary amide (XIII*b*, R = Me), purified by chromatography on alumina. A route to the *N,N*-dimethylamide that avoids the use of gaseous dimethylamine (39) was investigated earlier as applied to cholesterol. In this process, the crude acid chloride derived from cholesteryl acid succinate was heated with dimethylformamide. On cooling, the desired tertiary amide crystallized. Its infrared spectrum had peaks at 5.8 μ (C=O, ester) and 6.1 μ (C=O, *tert*-amide). However, this method failed with testosterone acid succinate.

The tetrahydropyranyl ether of testosterone (XIV) was prepared by the method of Ott and co-workers (23). Dihydropyran and a catalytic amount of *p*-toluenesulfonic acid were added to a suspension of testosterone in ether. Further quantities of dihydropyran and catalyst were added periodically over 3 weeks, during which time the testosterone completely dissolved.

There are a number of references to phenolic methoxymethyl ethers (13–16) prepared by treating sodio-derivatives of phenols with chloromethyl methyl ether. It was considered convenient to prepare the sodio-derivative of cholesterol by means of sodium hydride (available as a 50% dispersion in mineral oil). Cholesterol in benzene was heated with sodium hydride, the mixture cooled, and 20% excess of chloromethyl methyl ether added. Crystalline material was isolated from the organic phase that had an infrared spectrum consistent with the desired ether (absence of absorption in the O—H region and a peak at 8.64 μ attributable to C—O—C). The elemental analysis, however, was inconsistent with the methoxymethyl ether formulation (XVI). Of the possible alternatives considered, dicholesteryl formal (XV) was in accord with the analytical and spectral data. The result of a molecular weight determination (Rast) lent further support to the formal structure. Montignie (40) isolated a product that he claimed to be dicholesteryl formal, after shaking an ethereal solution of cholesterol with formaldehyde and sulfuric acid. No analysis was given, and the reported melting point was 50° lower than the product obtained in the present work. The procedure of Montignie (40) was repeated, and chromatographic purification of the product gave material with an infrared spectrum identical to that of our material and which did not depress its melting point. Therefore, it appears established that the product obtained by treating cholesterol with sodium hydride, followed by chloromethyl methyl ether, is dicholesteryl formal. Probably this is produced by the ready condensation of cholesterol (2 moles) with formaldehyde, derived during work-

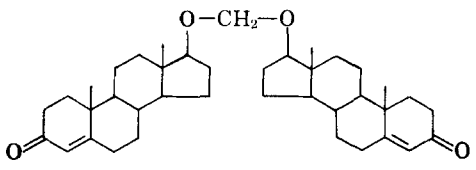


XIV

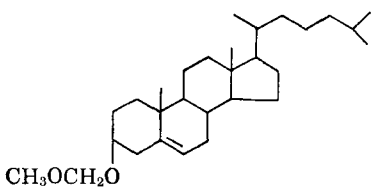


XV*a*

up from the hydrolysis of chloromethyl methyl ether. The isolation of cholesteryl formal from a mixture of cholesterol and chloromethyl methyl ether in benzene that had been shaken with water supports this contention. Under conditions similar to those used with cholesterol, testosterone also yielded a formal derivative; since this derivative potentially may yield testosterone *in vivo*, it was submitted for pharmacological evaluation. The reason for difficulty in preparing the methoxymethyl ether of cholesterol appears to be a lack of reaction between cholesterol and sodium hydride rather than instability of the methoxymethyl ether once formed, since treatment of the sterol with sodium hydride, followed by methyl iodide, gave starting material rather than the methyl ether.



XV*b*

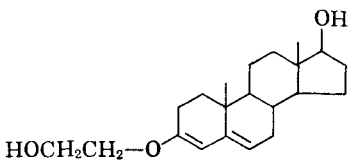


XVI*a*

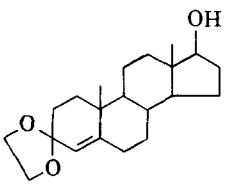
Stevens and Deans (41) used sodium naphthyl in dimethoxyethane to form the sodio-derivatives of various alcohols, including cholesterol; treatment of these salts with alkyl halides gave ethers. Cholesterol was treated with sodium naphthyl, as described, then with the theoretical amount of chloromethyl methyl ether. Stevens (41) removed naphthalene from the products by steam distillation; this method was considered too severe in the present case, and chromatographic separation on alumina was attempted. Naphthalene was eluted rapidly from the column by petroleum ether; elution with 25% benzene in petroleum ether gave the desired ether (XVI*a*) in good yield.

Due to the possibility of 1:2 and 1:4 addition of sodium naphthyl to a Δ^4-3-keto system, the method of Stevens and Deans (41) was applied to testosterone dioxolane (XVII) rather than to testosterone itself and the 17-methoxymethyl derivative (XVI*b*) isolated. Regeneration of the Δ^4-3-keto system in the ether (XVI*b*) by use of either acetone or pyruvic acid (*p*-toluenesulfonic acid catalyst in both cases) resulted in concomitant breakdown of the 17-methoxymethyl group. However, it was considered of interest to examine the biological properties of the dioxolane derivative (XVI*b*) itself since both pharmacodynamic groups potentially may be regenerated *in vivo*.

A by-product was isolated during the preparation of testosterone dioxolane (XVII), when this derivative was prepared by the method of Antonucci *et al.* (24) (1 mole of testosterone, 23 moles of ethylene glycol, *p*-toluenesulfonic acid catalyst in benzene). Use of a Dean-Stark water trap attached to the reaction flask represented a minor modification of the original method. Crystallization of the reaction product from either acetone or isopropyl ether–pyridine gave material, in 15% gross yield, of higher melting point than the dioxolane (XVII), with which by analysis it was shown to be isomeric. Apart from a more pronounced absorption band near 6 μ (C=C), the infrared spectrum of the isomer did not differ significantly from that of authentic dioxolane (XVII); its ultraviolet spectrum showed end absorption only. These spectral data together with the fact that it formed a monoacetylated product eliminated the enol ether (XVIII) as a possible formulation. Evidence that the by-product is the Δ^4-dioxolane (XIX) is provided by the fact that testosterone is regenerated from both isomers after acetone–*p*-toluenesulfonic acid treatment, and by

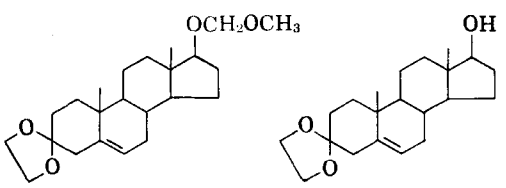


XVIII



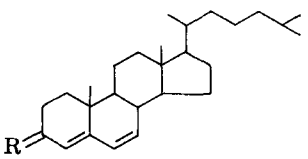
XIX

optical rotatory data described below. Conversion of Δ^4-3-ketosteroids to dioxolanes results in pronounced *levo* shifts in optical rotatory power. That these shifts are due in large part to ring A to B double bond migrations is evident from many examples in the literature (42). Furthermore, conversion of $\Delta^{4,8}$-cholestadiene-3-one (XX*a*) to its dioxolane (XX*b*) does not result in a *levo* shift in rotatory power, and it has been established that no double bond migration occurs in this reaction (43). On these grounds, the observed optical rotatory



XVI*b*

XVII



XX*a*, R=O

XX*b*, R=

TABLE I.—EFFECT OF ANDROGENIC-MYOTROPHIC ACTIVITIES IN RATS OF SEVERAL TESTOSTERONE ESTERS AND RELATED DERIVATIVES ADMINISTERED ORALLY

No.	Compd.	Mean % Increase in Seminal Vesicle Wt. over Castrated Control Rats (Androgenic Activity)	Mean % Increase in Levator Ani Wt. over Castrated Controls (Myotrophic Activity)
	Testosterone	47[a]	...
	Methyltestosterone	45[a]	19[b]
II	Testosterone pyruvate	27[a]	...
X	Testosterone acid succinate	28[a]	8
XII	Ethyl ester of testosterone acid succinate	30[b]	...
XIII*a*	Amide of testosterone acid succinate	35[a]	15
XIII*b*	*N,N*-Dimethylamide of testosterone acid succinate	30[a]	...
XI	Testosterone acid glutarate	17	...
XIV	Testosterone tetrahydropyranyl ether	45[a]	...
XVI*b*	Methoxymethyl ether of testosterone ethylene ketal	35.5[a]	26[a]
XV*b*	Ditestosteryl formal	7.5	...

[a] $p < 0.01$, Treatment group *versus* castrate control. [b] $p < 0.05$, Treatment group *versus* castrate control.

power of the dioxolation by-product from testosterone indicates that its formation does not involve bond migration—hence supporting formulation (XIX) for its structure. The NMR spectrum of the isomeric acetate also was consistent with assignment of a Δ^4-dioxolane structure. In particular, the signal for the vinylic proton (at C-4) was a singlet and was downfield relative to the multiplet due to the vinylic proton (at C-6) of the Δ^5-dioxolane. The NMR spectra of the deuteriochloroform solutions of the two dioxolane acetates, examined after storage for several weeks at room temperature, showed that equilibration had occurred; signals due to both forms (present in approximately equal amounts) were present. After completion of the chemical work reported here, Dean and Christiansen (44) reported isolation of the high melting ethylene ketal of testosterone and presented evidence in support of the same structural assignment (XIX).

The androgenic and myotrophic evaluation tests on these compounds related to testosterone were conducted to establish whether a derivative possessed activity and the degree of its activity when administered orally. Positive results would indicate absorption by the oral route. The magnitude of the positive values would give clues to potency relative to testosterone and methyltestosterone, but data relevant to duration of action would not be furnished by these tests.

As may be observed from the data in Table I, all of the testosterone derivatives, except for the glutarate ester and ditestosteryl formal, exhibited a significant ($p < 0.05$) degree of androgenic activity under the test conditions, manifested by increases in the weights of the seminal vesicles over those exhibited by the castrate controls. Though most of the new compounds exhibited androgenic activity, only the methoxymethyl ether of testosterone dioxolane exhibited significant myotrophic activity comparable to that of methyltestosterone. The amide of testosterone acid succinate and the testosterone acid succinate exhibited a slight degree of myotrophic stimulation. None of the steroids, when administered in oral aqueous suspension, possessed androgenic or myotrophic activity significantly greater than that exhibited by a comparable dose of methyltestosterone administered orally.

Of the seven compounds exhibiting appreciable androgenic activities, none appear to be more potent than testosterone or methyltestosterone at a 25 mg./Kg. dosage level. Administration of the testosterone derivatives at doses equimolar with those of testosterone perhaps would have resulted in data showing slight increases in their androgenic activities; however, the molecular weights of most of the derivatives are not appreciably different from testosterone. Therefore, conclusions from results of these studies perhaps would not be affected by administration of equimolar doses.

The closeness in magnitude of the androgenic activity values for the pyruvate, acid succinate, ethyl succinate, amidosuccinate, *N,N*-dimethylamidosuccinate, the tetrahydropyranyl ether, and the methoxymethyl ether derivative suggest strongly that most of these are latent forms of testosterone which, upon ingestion, are converted to testosterone. This surmise is supported further by the fact that, like testosterone, most of the compounds failed to exhibit significant myotrophic (anabolic) activity when administered orally.

Only the methoxymethyl ether of testosterone ethylene ketal appeared to exhibit significant myotrophic activity. Its androgenic and myotrophic activities closely mimicked those of methyltestosterone.

Ditestosteryl formal was almost devoid of *in vivo* activity, perhaps because it is poorly soluble in water and poorly absorbed.

REFERENCES

(1) Harper, N. J., *J. Med. Pharm. Chem.*, **1**, 467(1959).
(2) Harper, N. J., "Progress in Drug Research," vol. 4, Interscience Publishers, Inc., New York, N. Y., 1962, pp. 221–294.
(3) Brodie, B. B., and Hogben, C. A. M., *J. Pharm. Pharmacol.*, **9**, 345(1957).
(4) Williams, R. T., "Detoxification Mechanisms," Chapman and Hall, London, England, 1959.

(5) Reifenstein, E. C., Jr., *et al.*, *J. Am. Geriat. Soc.*, **2**, 293(1954).
(6) Ingold, C. K., "Structure and Mechanism in Organic Chemistry," Bell and Sons, London, England, 1953.
(7) Hammett, L. P., "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940.
(8) Sudborough, J. J., *J. Chem. Soc.*, **101**, 1227(1912).
(9) Bender, M. L., *Chem. Rev.*, **60**, 53(1960).
(10) Morawetz, H., and Westhead, E. W., Jr., *J. Polymer Sci.*, **16**, 273(1955).
(11) Morawetz, H., and Gaetjens, E., *ibid.*, **32**, 526(1958).
(12) Phillips, A. P., *J. Am. Chem. Soc.*, **75**, 4725(1953).
(13) Reychler, A., *Bull. Soc. Chim.*, **1**, 1195(1907).
(14) Pauly, H., and Wascher, K., *Ber.*, **56**, 603(1923).
(15) Rapoport, H., Baker, D. R., and Reist, A. N., *J. Org. Chem.*, **22**, 1489(1957).
(16) Robertson, D. N., *ibid.*, **25**, 931(1960).
(17) Bender, M. L., *J. Am. Chem. Soc.*, **84**, 2582(1962).
(18) Niemann, C., *Science*, **143**, 1287(1964).
(19) Davies, D. R., and Green, A. L., *Advan. Enzymol.*, **20**, 283(1958).
(20) Van Duin, H., *Rec. Trav. Chim.*, **73**, 78(1954).
(21) Ruzicka, L., and Wettstein, A., *Helv. Chim. Acta*, **19**, 1141(1936).
(22) Page, I. H., and Rudy, H., *Biochem. Z.*, **220**, 304 (1930).
(23) Ott, A. C., Murray, M. F., and Pederson, R. L., *J. Am. Chem. Soc.*, **74**, 1239(1952).
(24) Antonucci, R., *et al.*, *J. Org. Chem.*, **17**, 1341(1952).
(25) Eisenberg, E., and Gordan, G. S., *J. Pharmacol. Exptl. Therap.*, **99**, 38(1950).
(26) Carré, P., and Jullien, P., *Compt. Rend. Acad. Sci.*, **202**, 1521(1936).
(27) Hyman, M., Jr., and Johnson, P. H., Jr., U. S. pat. 2,440,092 (April 20, 1948).
(28) Lapkin, I. I., and Golovkova, A. I., *J. Gen. Chem. U.S.S.R.*, **18**, 485(1948).
(29) Weissberger, A., and Kibler, C. J., "Organic Syntheses," Coll. Vol. III, John Wiley & Sons, Inc., New York, N. Y., 1955, p. 610.
(30) Vanderhaeghe, H., *et al.*, *J. Am. Chem. Soc.*, **74**, 2810(1952).
(31) Marshall, C. W., *et al.*, *ibid.*, **70**, 1837(1948).
(32) Hershberg, E. B., *J. Org. Chem.*, **13**, 542(1948).
(33) McGuckin, W. F., and Kendall, E. C., *J. Am. Chem. Soc.*, **74**, 5811(1952).
(34) Ercoli, A., and Gardi, R., *ibid.*, **82**, 746(1960).
(35) Serini, A., and Koster, H., *Ber.*, **71**, 1766(1938).
(36) Nussbaum, A. L., *et al.*, *J. Org. Chem.*, **26**, 3925 (1961).
(37) Rosenkrantz, H., and Gut, M., *Helv. Chim. Acta*, **36**, 1000(1953).
(38) Hancock, J. E. H., and Linstead, R. P., *J. Chem. Soc.*, **1953**, 3490.
(39) Coppinger, G. M., *J. Am. Chem. Soc.*, **76**, 1372(1954).
(40) Montignie, E., *Bull. Soc. Chim. France*, **47**, 467 (1930).
(41) Stevens, P. G., and Deans, S. A. V., *Can. J. Res.*, **17B**, 290(1939).
(42) Fieser, L., and Fieser, M., "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959.
(43) Fonken, G. J., *J. Org. Chem.*, **26**, 2549(1961).
(44) Dean, J. W., and Christiansen, R. G., *ibid.*, **28**, 2110 (1963).
(45) Koshland, D. E., Jr., *Science*, **142**, 1533(1963).

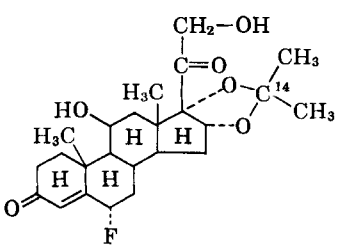
Thin-Layer Chromatographic Stability Assay for C^{14}-Labeled Steroid in a Cream

J. P. COMER and P. E. HARTSAW

The acetonide of 6α-fluoro-16α-hydroxyhydrocortisone, prepared from C^{14}-labeled acetone, was formulated as a cream. Initial assays were made by a conventional tetrazolium method and also by a thin-layer chromatographic separation and subsequent scintillation measurement of the C^{14}-labeled steroid. The cream samples were aged under accelerated conditions, and subsequent assays by the two methods were compared to the initial results. Small assay interferences caused by the aging of the formulation were demonstrated on the tetrazolium method. The techniques used illustrated advantages of the thin-layer chromatographic scintillation method for the early evaluation of a proposed stability method for a cream formulation.

THIN-LAYER chromatographic techniques have been useful for the quantitative steroid assay as drug substances (1) and in stability assay of aqueous solutions (2). Common measurements of the eluted steroids are by ultraviolet spectrophotometric or tetrazolium colorimetric procedures. Formulation and stability studies of cream preparations showed that placebo preparations, on accelerated aging, sometimes gave positive interference by conventional techniques. It was noted also that in some preparations aging increased binding of the steroids, with a resultant loss of steroid during extraction.

The method reported here was designed to evaluate a proposed conventional stability method to determine whether the errors mentioned would be significant on aged samples of a cream formulation of the acetonide of 6α-fluoro-16α-hydroxyhydrocortisone (I). The cream containing the C^{14}-labeled steroid was prepared by normal procedures and placed in accelerated aging conditions. The stability method under study involved the red tetrazolium colorimetric assay of a chloroform extract of the steroid cream along with the cream placebo.



6α-Fluoro-16α-hydroxyhydrocortisone-16,17-acetonide

I

Received August 26, 1964, from the Analytical Development Department, Eli Lilly and Co., Indianapolis, Ind.
Accepted for publication December 21, 1964.
Presented to the Scientific Section, A.PH.A., New York City meeting, August 1964.