Conversion of a C-20-Deoxy-C_{21} Steroid, 5-Pregnen-3β-ol, into Testosterone by Rat Testicular Microsomes*

(Richard B. Hochberg, Shoshana Ladany, and Seymour Lieberman)

From the Departments of Biochemistry and of Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University, New York, New York 10032

5-[7α-^3H]Pregnen-3β-ol, a C-20-deoxy analog of pregnenolone, was synthesized and tested as a substrate for the enzyme system occurring in testes that cleaves the side chain of C_{21} steroids between C-17 and C-20. This C-20-deoxy C_{21} steroid was incubated with a microsomal preparation obtained from rat testis and was converted into testosterone in 5% yield. Another C-20-deoxy analog of pregnenolone, 5,20-pregnadien-3β-ol, was not converted into testosterone by this enzyme system. The significance of this finding for the natural processes by which pregnenolone is converted by the same subcellular fraction into the male sex hormone is examined in the light of the hypothesis that intermediates involved in steroidogenesis are transient, reactive complexes of the appropriate reactants (steroids, oxygen, etc.) with specific enzymes.

Schemes for the pathways by which the steroid hormones are biosynthesized are derived from several kinds of evidence. Chemical intuition based on known chemical reactions suggests entirely plausible routes. Such rationalizations, when fortified by experimental evidence coming from the isolation of well characterized, endogenous, steroidal metabolites from urine, led to the formulation of step-wise, discrete reaction sequences by means of which cholesterol is converted into the various steroid hormones. When steroids labeled with ^3H or ^14C became available, it became easy to test whether suspected intermediates were converted by in vitro experiments using endocrine gland preparations into expected end-products. Thus the schemes created from these kinds of evidence portrayed the transformation of cholesterol to the hormones as proceeding through a series of stable, isolable hydroxylated intermediates which at proper stages were fragmented through the intervention of enzymes which cleave C=C bonds (C_{21} + C_{20} + C_{19} + C_{18} + C_{17} + C_{16} + C_{21} + C_{20} + C_{19} + C_{18} + C_{17} + C_{16} + C_{21}). Such sequences accounted for the biosynthesis of the hormones progesterone, testosterone, and estradiol merely by adding the proper dehydrogenases and isomerases required to convert the 3β-hydroxy-5-ene structures present in Ring A of cholesterol and of pregnenolone into the 3-oxo-4-ene group characteristic of progesterone and testosterone. For the biosynthesis of cortisol and aldosterone, specific hydroxylated derivatives of progesterone are shown in these outlines as stable intermediates. In short, these schemes imply the existence of stable, isolable compounds as obligatory intermediates. The supposition is that each reaction in the sequence yields a product characterized by a well recognized chemical structure and that then this product undergoes the next reaction in the sequence affording the next compound in the chain. The pathway is followed until the end-product is formed. Implied in these models is the notion that all intermediates are intermixed in some random way so that, for example, a molecule of 17α-hydroxyprogesterone destined to become cortisol in the adrenal is indistinguishable in structure or location from that molecule that will be used to produce androstenedione or testosterone in that gland. Of course it is well known that the enzymes catalyzing some of these reactions are localized in mitochondria (cholesterol-side chain lystate, 11β- and 18-hydroxylase), and others reside in microsomes (e.g. 17- and 21-hydroxylases, C_{17}-C_{21} lyase, aromatase) so that such a simple representation cannot be true.

Recently we have proposed that steroid hormones are manufactured in "biosynthetic units" consisting of multi-enzyme complexes in which all the components for the complete synthesis of a hormone from its sterol precursor are present and arranged in such a way that the substrates associated to appropriate enzymes are converted by a series of concerted reactions into the proper end-product (1). In this view the true intermediates are not stable isolable compounds but are...
transient, enzyme-bound species. The hydroxylated steroids that can serve as precursors in in vitro experiments or that can be isolated from these experiments or from tissues or urine, are considered, according to this hypothesis, to be by-products formed inadvertently, by hydrolysis, from the substrate enzyme complexes.

The experimental evidence that formed the basis for this hypothesis came from experiments in which synthetic analogs of cholesterol were used as substrates for the mitochondrial reaction: cholesterol → pregnenolone + isocaproic aldehyde (2, 3). These analogs were so constituted that the carbon atom cleaved from C-20 was completely substituted or was part of an aromatic ring and thus could not be hydroxylated, or at least not without simultaneous cleavage of the C-20-C bond. When incubated with preparations of mitochondria from bovine adrenal cortex, these analogs were readily cleaved and formed pregnenolone, results which led to the suggestion that the step-wise formation of the discrete intermediates, 20α-hydroxycholesterol and more particularly, the glycol, 20α,22R-dihydroxysterol, was not obligatory for the conversion of cholesterol to pregnenolone.

In this paper results are presented which may be taken to add further support for this hypothesis. Here the formation of the C₁₉-androsterone, testosterone, from a 20-deoxy analog of pregnenolone is demonstrated using microsomes from rat testis. In the traditional view, the C₁₉ androgens are formed from pregnenolone through the intermediacy of a 17α-hydroxy-20-keto compound, 17α-hydroxyprogesterone. That this compound or its relative, 17α-hydroxyprogrenolone, can serve as exogenous precursors for C₁₉ steroids is now well known for about 25 years (4). These compounds have been isolated from steroidalogenic tissue (5), blood (6), and urine (7) and, in addition, their formation in in vitro experiments from progesterone or pregnenolone has been demonstrated (8). These results led to the presumption that compounds like them, having oxygen functions on vicinal carbon atoms, are obligatory intermediates for the oxidative cleavage of the 2-carbon side chain of C₁₉ steroids. Since our previous experience with analogs of cholesterol (2, 3) showed that the 20,22-glycol was not an essential intermediate in the side chain cleavage process, we attempted to test whether a stable C₁₉ compound having oxygen atoms on C-17 and C-20 was required for the conversion in which a C₁₉,O₂ product is formed by fission of the Cl₉-androgen, testosterone, from a 20,22-deoxy analog of pregnenolone, was used as a substrate for the enzymes present in testicular microsomes. No testosterone was formed from this olefinic compound.

EXPERIMENTAL PROCEDURE

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra were taken in micro potassium bromide discs with a Perkin-Elmer model 521 infrared spectrophotometer equipped with dual 4x reflecting beam condensors. Nuclear magnetic resonance spectra were determined with a Varian HA-100 spectrometer. Mass spectra were obtained with a Du Pont 21-492B mass spectrometer and a computerized data system. Radioactive samples were counted in a Packard model 307 liquid scintillation spectrometer with a liquid scintillation efficiency of 63% for 3H and a counting error of less than ±0.5%. A Waters ALC 100 liquid chromatograph equipped with a 254 nm differential ultraviolet detector and a differential refractometer was used for high pressure liquid chromatography. Partition column chromatography was done as described by Siiteri (9).

AgNO₃-silica gel (20% AgNO₃, w/w) was prepared by mixing silica gel and AgNO₃ in a 2:1 ratio. This mixture was stirred and heated in an oven at 100°C for 2 h. The mixture was then cooled to room temperature and used as the stationary phase. All solvents were reagent grade. The mobile phase was a mixture of methanol-ethyl ether and acetonitrile. The resulting 3H-pregnenolone was eluted from New England Nuclear Corp. It was acetylated and purified by chromatography and crystallization.

Pregnenolone acetate was eluted by a mixture (7/3) of benzene/petroleum ether. The resulting 3H-pregnenolone was converted into its 3-methoxime and this, too, was purified by chromatography and crystallization. The yield of testosterone was calculated from the amount of tritium associated with the carrier to be 4.3%. The yields from three other experiments using different samples of microsomes, averaged 2%.

In an effort to extend these observations, another deoxy analog of pregnenolone, 5,20-pregnadien-3β-ol, was used as a substrate for the enzymes present in testicular microsomes. No testosterone was formed from this olefinic compound.
Androgen Biosynthesis from a C-20-Deoxy-C$_2$, Steroid

Pregnenolone tosylhydrazone was prepared as described previously (13). The tosylhydrazide was dissolved in tetrahydrofuran and was treated at room temperature with 2 ml of butyllithium using the procedure described by Shapiro and Heath (14). The steroid diene was crystallized twice from a mixture of acetone and methanol, once from methanol and once from acetone. The resulting product melted at 338-339$^\circ$C [d$^0$ = -84.9 to -85.0$^\circ$] in 5 ml of CHCl$_3$; reported (15) m.p. 130-130.5$^\circ$C, [a]$^0_{D}$ = -65$^\circ$ to +1$^\circ$. Infrared spectrum (micro KBr) 3380 (broad, 3$'$-OH), 3070 (C=CH$_2$); nuclear magnetic resonance spectrum (CDCl$_3$, tetramethylsilane): $\delta$ 5.76 (m, 1, 11-20), 5.37 (t, 1, 11-0), 5.04 (d, 1, 11-21 cis to H-20), 4.9 (q, 1, 11-21 trans to H-20), 3.54 (m, 1, H-30), 1.01 (s, 3, H-19), and 0.60 cpm (s, 3, H-18); mass spectrum (76 eV): 300 (100, parent and base), 285 (20.4, M-CH$_3$), 252 (20.3, M-H$_2$O), 267 (77.4, M-H$_2$O-CH$_3$), 213 (37.7), 145 (26), 143 (24.5), 119 (30.2), 107 (28.4), 105 (45.3), and 91 (54.7).

Pregnenolone acetate (2.4 x 10$^5$ cpm) was treated with 0.1 ml of a solution containing 7% (v/v) ethanethiol and 1.7% (w/v) of p-toluensulfonic acid in glacial acetic acid (16). The reaction mixture was kept overnight at room temperature. Ether (6 ml) was added and the solution was washed three times with 1-ml portions of a 0.2 M NaOH solution and then twice with distilled water. A drop of glacial acetic acid was added with the final aqueous wash and the solution was taken to dryness under vacuum at room temperature. The dry residue was chromatographed on a silica gel thin layer plate using benzene as eluent. (Rf values: pregnenolone-3-acetate-20-ethylenethioketal, 0.46; pregnenolone acetate, 0.25). The zone corresponding to the reference sample of pregnenolone-3-acetate-20-ethylenethioketal contained 1.6 x 10$^5$ cpm. The product was eluted from the absorbent with ether. The solvent was evaporated at room temperature under reduced pressure and the residue was desulfurized with W-2 Raney nickel in ethanol solution (3). Following saponification of the product, the resulting alcohol was chromatographed on a 4 g AgNO$_3$-silica gel column. The major product containing 1 x 10$^5$ cpm of $^3$H was eluted by a 1/1 mixture of benzene/petroleum ether. Possible impurities are 5,20-pregnadien-3-ol and pregnenolone-20-ethylenethioketal, but the former is eluted from the AgNO$_3$-silica gel column with a 2/1 mixture of benzene/petroleum ether, while the latter compound was not eluted even with a 4/1 mixture of benzene/petroleum ether. After evaporation of the solvent, the radioactive residue was chromatographed by high pressure liquid chromatography on a Bondapack C$_18$/Corasil II column (3 feet x 1/8 inch) using acetonitrile/water (9/1) as the mobile phase, with a flow rate of 0.5 ml/min. The product containing 0.78 x 10$^5$ cpm of $^3$H was eluted after 15.6 min. The retention times of authentic 5,20-pregnadien-3-ol and 5-pregnene-31-ol are, under these conditions, 15.6 and 9.6 min, respectively.

To confirm the identity and radiochemical purity of the tracer, a portion of the radioactive material was mixed with unlabeled 5-pregnene-31-ol and subjected to high pressure liquid chromatography as described above. The curve describing the elution of radioactivity coincided with that related to the mass of the product as determined by its refractive index. Another portion was diluted with unlabeled 5-pregnene-31-ol and the mixture was recrystallized several times. The data in Table II show that the initial specific activity of the mixture was maintained throughout three crystallizations. That the specific activity of the product from the first recrystallization was the same as that expected of the original mixture of the labeled material diluted with carrier proved that no contaminant was present in the original sample of the radioactive tracer.

The presence of even a trace of highly radioactive pregnenolone, the compound from which 5-pregnene-31-ol was prepared, could invalidate any conclusion based on the demonstration of the formation of testosterone, since the latter hormone would naturally be produced from pregnenolone. Therefore a special effort was made to determine the maximum possible contamination of 5-pregnene-31-ol with pregnenolone. This estimate was made using the devise of a "wash out" experiment. A trace sample of 5-$^3$H-pregnenolone-31-ol (i.e., x 10$^5$ cpm of $^3$H) was diluted with 10.9 mg of unlabeled pregnenolone and the mixture was chromatographed on Celite in System II. In this system, the tracer migrated in the first part of the 1st holdback volume and pregnenolone is eluted in holdback volumes 6 to 7. The contents of those tubes containing pregnenolone were combined, the solvent evaporated and the amount of tritium present was determined. In all, 1.877 cpm were present, the specific activity of the residue was 167 cpm/mg. After one crystallization from acetone, the specific activity of the crystalline pregnenolone was reduced to 7.5 cpm/mg (81.5 cpm in total sample). Thus, at most 0.007% of the tritium associated with the tracer sample of 5-$^3$H-pregnenolone-31-ol could have been present as pregnenolone.

Pregnenolone and pregnenolone acetate were synthesized from 5-carbon steroid precursors as described (41.2), 121 (39.2), 120 (39.0), 119 (41.1), 109 (29.2), 107 (19.0), 105 (80.8), 93 (70.4), and 91 (100.0, base).
Androgen Biosynthesis from a C-20-Deoxy-C_{21}, Steroid

Dry tetrahydofuran and 0.2 ml of a solution of 1.9 M methyl lithium in ether (Alfa Co.) was added. The mixture was stirred at room temperature for 3 hours. After the addition of 2 ml of water the product was extracted from the aqueous mixture first with 10 ml of petroleum ether and then 10 ml of ethyl ether. The organic phases were combined and washed until neutral with small portions of water. The organic solvents were evaporated and the residue was chromatographed on an AgNO\textsubscript{3}-silica gel column. The diene was eluted with a 3:1 mixture of benzene/petroleum ether. The fractions containing the tracer were chromatographed on the preparative layer chromatograph in the system: benzene/hexane/ethyl acetate. The homogenate was centrifuged at 360,000 × g for 45 min. The supernatant was centrifuged at 105,000 × g for 90 min to yield the microsomal pellet. It was suspended in the isolation medium (0.5 to 1 ml/pair of testes) and used as such in subsequent incubations.

In Vitro Conversion of 5\textdagger\textsterisk{20}-Deoxy-5\textdagger\textsterisk{21}-pregnen-3\textsterisk{18}-ol to Testosterone

A solution of the radioactive substrate dissolved in benzene/ethanol (95/5) was introduced into a test tube (15 × 100 mm). The solvent was evaporated under a stream of N\textsubscript{2} and the residue was dissolved in 0.02 ml of ethanol. One-half milliliter of 0.1 M phosphate buffer, pH 7.4, containing a NADPH-generating system, 0.31 mg (0.4 pmol) of NADP, 0.91 mg (3 µmol) of glucose 6-phosphate, and 2 units of glucose 6-phosphate dehydrogenase (Sigma Chemical Co.) and 1 µmol of MgCl\textsubscript{2} were added. The mixture was preincubated in an agitating water bath at 34°C for 10 min. Finally, 0.5 ml of the microsomal suspension described above containing, on average, at least 10 mg of protein, (17) was added and the incubation mixture was shaken at 34°C for 45 min. The reaction was stopped by the addition of methanol (5 to 10 ml) containing the appropriate carrier steroids. The aqueous methanol solution was filtered, and the precipitate was washed thoroughly with methanol and then with benzene. The combined filtrates were evaporated to dryness and the residue was chromatographed as described below.

In Vivo Conversion of 5\textdagger\textsterisk{20}-Deoxy-5\textdagger\textsterisk{21}-pregnen-3\textsterisk{18}-ol to Testosterone

5\textdagger\textsterisk{20}-[\textdagger\textsterisk{3}H]\textsterisk{21}-pregnen-3\textsterisk{18}-ol (2.07 × 10\textsuperscript{3} cpm, 66.2 pmol) was incubated according to the procedure described above. After incubation, the following carrier steroids dissolved in methanol were added: testosterone, 5 mg; pregnenolone, 5-pregnene-3\textsterisk{18},20\textdagger\textsterisk{18}-diol, and 5-pregnene-3\textsterisk{18},20\textdagger\textsterisk{18}-dihydroxy. The mixture was filtered and washed neutral with small portions of water. The organic solvents were evaporated and the residue was chromatographed on a AgNO\textsubscript{3}-silica gel column. The diene was eluted with a 3:1 mixture of benzene/petroleum ether. The fractions containing the tracer were chromatographed on the preparative layer chromatograph in the system: benzene/hexane/ethyl acetate. The homogenate was centrifuged at 360,000 × g for 45 min. The supernatant was centrifuged at 105,000 × g for 90 min to yield the microsomal pellet. It was suspended in the isolation medium (0.5 to 1 ml/pair of testes) and used as such in subsequent incubations.

Preparation of Rat Testes Microsomes

Adult male Sprague-Dawley rats, weighing 200 to 250 g were injected subcutaneously with 10 IU of HCG (Ayerst, A.P.L.C. chionic gonadotropin in aqueous solution) daily for 4 consecutive days prior to sacrifice. On the day of sacrifice, the rats were killed by severing the spinal cord. Testes were removed immediately, stripped of their capsules and put into ice-cold isolation medium consisting of 0.25 M sucrose and 0.01 M EDTA. Subsequent procedures were carried out at 0-4°C. When the decapsulated testes from 3 to 6 rats had been collected, blotted on filter paper, weighed and quartered, a 15% homogenate (w/v) of the testes was prepared in the isolation medium. The homogenate was centrifuged for 10 min at 800 × g and the supernatant was again centrifuged at 10,500 × g for 25 min. The resulting supernatant was centrifuged at 105,000 × g for 90 min to yield the microsomal pellet. It was suspended in the isolation medium (0.5 to 1 ml/pair of testes) and used as such in subsequent incubations.

Incubation: General Procedure

A solution of the radioactive substrate dissolved in benzene/ethanol (95/5) was introduced into a test tube (15 × 100 mm). The solvent was evaporated under a stream of N\textsubscript{2} and the residue was dissolved in 0.02 ml of ethanol. One-half milliliter of 0.1 M phosphate buffer, pH 7.4, containing a NADPH-generating system, 0.31 mg (0.4 pmol) of NADP, 0.91 mg (3 µmol) of glucose 6-phosphate, and 2 units of glucose 6-phosphate dehydrogenase (Sigma Chemical Co.) and 1 µmol of MgCl\textsubscript{2} were added. The mixture was preincubated in an agitating water bath at 34°C for 10 min. Finally, 0.5 ml of the microsomal suspension described above containing, on average, at least 10 mg of protein, (17) was added and the incubation mixture was shaken at 34°C for 45 min. The reaction was stopped by the addition of methanol (5 to 10 ml) containing the appropriate carrier steroids. The aqueous methanol solution was filtered, and the precipitate was washed thoroughly with methanol and then with benzene. The combined filtrates were evaporated to dryness and the residue was chromatographed as described below.

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the residues in the mother liquors were determined. All of the isolated carriers contained small amounts of tritium. Insufficient amounts of material prevented the purification of any of the products to constant specific activity or to the point where all the radioactivity was removed. The specific activities of the products crystallized the last time in System II were: pregnenolone, 13 cpm/mg; 5-pregnen-3β,20α-diol, 14 cpm/mg; and 5-pregnen-3β,20β-diol, 17 cpm/mg. This would correspond, respectively, to yields of 0.01%, 0.01%, and 0.01%.

**Incubation of 5,20-Pregnadien-3β-ol**

5,20-pregnen-3β-ol (1.1 x 10^6 cpm, 0.04 pmol) was incubated with rat testicular microsomes exactly as described above. The carrier testosterone (5 mg) was reisolated by a single chromatogram on Celite in System II. After one crystallization from acetone/H2O, the crystalline product contained only 265 cpm corresponding to a specific activity of 53 cpm/mg. This indicates a maximum possible yield of 0.02%, which was considered to be insignificant. Further purification was not attempted.

**Control Incubations**—In each of the following experiments an aliquot of the same microsomal preparation was used.

1. **Bovine Microsomes**—5[[7-3H]Pregnen-3β-ol (2.07 x 10^5 cpm, 67.2 pmol) was incubated as described above with a TPNH-generating system and a 0.5 ml aliquot of a microsomal preparation which had previously been heated in a boiling water bath for 5 min. Methanol containing 5 mg of testosterone was added at the appropriate time and the solids were filtered. The filtrate was concentrated to dryness and the residue was chromatographed once on Celite in System II. The carrier testosterone (eluted in the 17th to 20th holdback volume) was crystallized once from acetone/petroleum ether (30-60%). The specific activity of the crystalline residue was 73 cpm/mg corresponding to a yield of 0.02%. Since this amount of radioactivity was considered to be insignificant, no further purification was attempted.

2. **Pregnenolone to Testosterone**—5[[7-3H]Pregnen-3β-ol (1.2 x 10^4 cpm, 68 pmol) was incubated with the preparation of testicular microsomes and the products were worked up as described above. After chromatography on Celite first in System II and then in System III and finally on a high pressure liquid chromatograph, the testosterone carrier was crystallized once. The specific activity of the product before the last chromatogram was the same as that observed after crystallization, 99,300 cpm/mg. This corresponded to a yield of 497,000 cpm or 27%.

3. **Cholesterol**—2,3,4,5,6-pregnenolone (1.2 x 10^5 cpm, 25 pmol) was incubated with the same microsomal preparation and the products extracted as described before. After one purification by chromatography on Celite in System II, the carrier testosterone was crystallized once from acetone/petroleum ether (30-60%). The specific activity of the crystals was 18 cpm/mg, corresponding to a maximum yield of testosterone of 80 cpm or 0.01%. No further purification was attempted.

**Incubation of 5-Pregnen-3β-ol with Testicular Mitochondria**

The 8,000 x g pellet from the original testicular homogenate was resuspended in 40 ml of isolation media containing 0.01 M Tris-HCl, pH 7.4. The suspension was centrifuged at 650 x g for 10 min and the supernatant was centrifuged at 8000 x g for 10 min. The resulting pellet was washed twice by resuspension in the isolation media, followed by centrifugation at 8000 x g for 10 min. The final pellet was dispersed in 1.7 ml of the isolation media which, in addition to its usual constituents, was 0.01 M with respect to CaCl₂. A 0.5 ml portion of this mitochondrial suspension, containing 45 mg of protein, was incubated with 2,3,4,5,6-pregnenolone (948,000 cpm, 31 pmol) and a TPNH-generating system as described for the microsomal preparation. Pregnenolone and 5-pregnen-3β,20α-diol, as well as 5-pregnen-3β,20β-diol, were added as carriers after the incubation was terminated. After chromatography on Celite in System II (pregnenolone was eluted in the 14th to 15th holdback volume, and 5-pregnen-3β,20α-diol was eluted in the 16th to 18th holdback volume), the reisolated carriers were crystallized once. The amount of radioactivity found in the crystalline products (calculated from the specific activities) were: pregnenolone, 420 cpm, 0.04%; 5-pregnen-3β,20α-diol, 375 cpm, 0.04%; 5-pregnen-3β,20β-diol, 260 cpm, 0.03%. These were considered to be insignificant and further purification was not attempted.

**DISCUSSION**

5-Pregnen-3β-ol was prepared by the Raney nickel desulfuration of the 20-ethylenethiolate derivative of pregnenolone. Special care was required to ensure that the radioactive tracer of this compound was not contaminated with tritiated pregnenolone from which it had been prepared. Since pregnenolone serves as an excellent precursor of testosterone, its presence even in small quantities in the sample of 20-deoxy-pregnenolone converted to the C₁₉ hormone would tend to invalidate the result. The "wash out" chromatogram proved that no radioactivity accompanied the elution of pregnenolone when a mixture of it and the tracer were separated by chromatographic analysis. This procedure is an extremely sensitive device for the detection of infinitesimal amounts of impurities which might fail to be excluded by other purification processes. Still another excellent method for establishing homogeneity of the tracer was used. It involved recrystallization of a sample of the tracer diluted with authentic carrier and the demonstration that the specific activities of the tracer and of the residue left in the mother liquor were identical. A trace amount of a highly radioactive contaminant, if present, would have increased the isotope content of the first mother liquor disproportionately.

In an attempt to understand the conversion of 5-pregnen-3β-ol to testosterone better, several control experiments were carried out. Heat-denatured microsomes were incapable of effecting the transformation, thus assuring the enzymatic nature of the reaction. No enzyme capable of catalyzing the hydroxylation of C-20 of a C₁₉ steroid has ever been reported, but if testicular microsomes possess such an enzyme the conversion reported in this paper (5-pregnen-3β,20β-diol → testosterone) could be readily explained. Formation of pregnenolone from 5-pregnen-3β-ol by C-20 oxygenation would lead to the production of testosterone since the C₁₉O₂ compound is, as shown by one of the control experiments, readily converted to the male sex hormone. Although enzymatic hydroxylation at C-20 occurs, the only known substrates for this reaction are cholesterol (18, 19) and some of its analogs (3, 19). Moreover, this 20-hydroxylase has been found only in the mitochondria. To search for a 20-hydroxylase in testicular microsomes, two control incubation experiments were carried out. In one, [3H]cholesterol was incubated with testicular microsomes and carrier testosterone was reisolated devoid of any radioactivity. If a C₁₉O₂ intermediate such as pregnenolone had been formed from cholesterol, it would readily have been converted into testosterone. This control proves that the preparation used by us possessed no cholesterol side chain activity and therefore no enzyme capable of C-20 oxidation.

In the other control, 5[[7-3H]pregnen-3β-ol was the substrate for testicular microsomes. A search was made for the formation of three possible C₁₉O₂ products by using pregnenolone and the 20α and 20β isomers of pregnenediol as carriers. None of the reisolated samples of these carriers contained a substantial amount of radioactivity. The small amount of tritium associated with these compounds, when the purification was terminated, would correspond to a yield of at most 0.03%. Even this minute quantity of isotope, however, introduces an uncertainty so that it is impossible to state categorically that pregnenolone (or a relative) was not an intermediate in the conversion of pregnenolone to testosterone. The formation of a C₁₉O₂ intermediate might be difficult to detect because it could be rapidly metabolized to testosterone in the preparations used.
Finally an effort was made to determine whether the cholesteryl side chain enzyme system existing in testicular mitochondria could employ 5-pregnen-3β-ol as a substrate for C-20 oxygenation. Neither pregnenolone nor either of its epimeric C-20,21-dihydro reduction products were formed when pregnenolone incubated with such mitochondria. In summary then, our results show that, although testicular microsomes can convert 5-pregnenol to testosterone, the subcellular preparatory cannot use cholesteryl as a precursor for any recognizable C₂₁ or C₁₉ steroids. No evidence has been obtained that shows that they convert pregnenolone into C₃,₉,₂₀ compounds, but this possibility has not been conclusively excluded. Thus it would appear that there are several possible explanations for our findings. According to our hypothesis, the true intermediates in steroidogenic processes are reactive complexes in which the steroidal substrate is bound to a metalloenzyme located on a structured biosynthetic unit. In the case of the reaction pregnenol → testosterone, one explanation would involve a complex in which the 20-deoxy-pregnenolone is bound to the metalloenzyme through oxygen linked at C-17. If this complex has properties resembling an alkoxy radical then it is easy to rationalize the reaction, since such species easily cleave by β-scission (between C₁₇ and C₁₉) and would yield the C₁₇-oxygenated steroid, testosterone. Our experiments do not permit a distinction between a radical intermediate and one involving ionic species or, as pointed out previously, such a distinction may be artificial, since a complex between the ligand and the metal may be considered to be a resonance hybrid which cannot be represented faithfully by any single valence bond structure. In this proposal, a second attack at C-20 is unnecessary for cleavage between C-17 and C-20 to occur.

Another alternative would involve oxygenation of pregnenolone at C-20. In the course of severing the side chain of colesterol, oxygenation of 2 adjacent carbon atoms, C-20 and C-22, appears to be required before fission takes place. If hydroxylation of C-20 of pregnenolone itself, or of pregnenolone associated to the metalloenzyme through oxygen at C-17, is necessary for fission, then the hydroxylase catalyzing the oxygenation at C-20 would be one that has not previously been reported.

Another possibility is one in which cleavage of the 2-carbon side chain of pregnenolone requires nucleophilic attack at C-20 but does not involve insertion of another atom of oxygen. In the natural process by which testosterone is formed from pregnenolone, 17-hydroxypregosterone, or better, its complex with some component of the biosynthetic unit, is cleaved probably by a reagent attacking at C-20. Whether this reagent is an enzyme that catalyzes the introduction of another atom of oxygen or whether it is one that reacts through another nucleophilic group is unknown. But in either case, this same enzyme could be the reactive (at C-20) species necessary for the side chain cleavage of the complex of pregnenolone and the 17-hydroxylase metalloenzyme. Acetic acid is the 2-carbon fragment formed when pregnenolone is metabolized to C₃,₉,₂₀ products (20) but whether one or both of its 2 oxygen atoms comes from molecular oxygen via a reaction involving hydroxylases is unknown. We are presently examining the side chain fragment derived from pregnenolone in the hope it will provide clues about the mechanism of cleavage of this analog.

There was a possibility that the tracer 5,20-pregnadien-3β-ol prepared by us possessed the wrong configuration at C-17 and that this was responsible for its failure to serve as a precursor of testosterone. Determination of its specific rotation appears to eliminate that likelihood, for both that and the melting point were identical to those reported by Julian et al. (15). The configuration of C-17 of the dienol prepared by these investigators was established by its conversion to the same 20,21-dihydroxy derivative that was formed by reduction of the C-20 carbonyl group of deoxycorticosterone. It seems reasonable to explain the failure of this dienol to serve as a substrate for this cleavage reaction by recognizing that a C=C group is a much poorer leaving moiety than either CH₃(CH₂)₃ or CH₃CO— (in the case of pregnenolone). Whatever the mechanism of cleavage, the formation of a reactive vinylic species, which is either radical or ionic in nature, during fission appears to be sufficiently difficult so that no reaction occurs.

Acknowledgment—The authors gratefully acknowledge the assistance of Dr. Patrick D. McDonald.

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