Studies on the Biosynthetic Conversion of Cholesterol into Pregnenolone

SIDE CHAIN CLEAVAGE OF A t-BUTYL ANALOG OF 20a-HYDROXYCHOLESTEROL, (20R)-20-t-BUTYL-5-PREGNENE-3β,20-DIOL, A COMPOUND COMPLETELY SUBSTITUTED AT C-22*

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SUMMARY

(20R)-20-t-Butyl-5-pregnene-3ß,20-diol, an analog of 20α-hydroxycholesterol, has been synthesized and its metabolism studied. Since C-22 in this synthetic compound is completely substituted, this position is unavailable for biological oxygenation. When injected intravenously into a rabbit, the t-butyl analog is metabolized to urinary pregnanediol. When incubated with sonicated mitochondria from bovine adrenal glands, it is converted into pregnenolone. Two mechanisms consistent with these findings are proposed; both predict the involvement of reactive, transient, intermediate complexes which are represented simply either as radical or ionic species. However, the actual mechanism may best be imagined to be some hybrid of these two extreme processes. These and other results form the basis of a new hypothesis for the pathways used for the biosynthesis of pregnenolone from cholesterol. In this scheme, the traditional side chain-hydroxylated compounds are not obligatory intermediates; rather, they are considered to be by-products resulting from competitive reactions of short lived, reactive species.

Based upon observations made during the last decade, cholesterol,¹ is generally considered to be converted into pregnenolone, 6, a precursor of all steroid hormones, by synthetic routes involving side chain-hydroxylated C27 intermediates: 20α-hydroxycholesterol, 2, 22R-hydroxycholesterol, 3, and the glycol, 20α,22R-dihydroxycholesterol, 4. An excellent and complete review of the subject was recently written by Sulimovici and Boyd (2). Each of these derivatives, 2, 3, and 4, when incubated with appropriate preparations of endocrine tissues, has been shown to be transformed into pregnenolone. In fact, each is converted into the C27 steroid at a rate faster than that of cholesterol. Further evidence bearing on their intermediary includes the fact that both 22R-hydroxycholesterol and the glycol have been isolated in crystalline form from extracts of adrenal glands (3). Detection of the trace amounts of 20α-hydroxycholesterol present in these extracts required indirect procedures (4). The glycol 4 has been thought to be an intermediate since it is obtained as a product during the incubation of the monohydroxylated derivatives 2 and 3 (5, 6) as well as of cholesterol (6, 7). Thus, from these results, these naturally occurring hydroxylated derivatives of cholesterol have been assumed to be obligatory intermediates in the biosynthetic process by which the C27 sterol is converted into the C21 ketone.

However, such a scheme does not seem entirely satisfactory in view of certain recent observations. If the above mentioned circumstantial evidence for the intermediacy of 2, 3, and 4 is

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¹ Systematic names for steroids (1) given trivial names in the text are: 1, 5-cholesten-3ß-ol; 2, 5-cholestene-3ß,20α-diol; 3, (22R)-5-cholestene-3ß,22-diol; 4, (22R)-5-cholestene-3ß,20α,22-triol; 5, 20α-hydroperoxy-5-cholesten-3ß-ol; 6, 3ß-hydroxy-5-pregnen-20-one; 7, 21 hydroxy 4 pregnene 3,20-dione; 8, 4,16-androstan-3-one; 9, 24-Nor-22,22-dimethyl-5-chole-3ß,20α-diol; 10, 5ß-pregnane-3α,20α-diol; pregnenedione, 5ß-pregnane-3ß,20-dione; pregnostenone, 4-pregnen-3ß,20-dione. Note: In the text, Compound 9 will be referred to as (20R)-20-t-butyl-5-preg-
deemed sufficient, then the existence of several pathways for the conversion of cholesterol into pregnenolone must be assumed. Simpson and Boyd (8) have already pointed out that this is an unlikely possibility. Moreover, Burstein et al. (9) have conducted an elaborate kinetic analysis of the pertinent reactions and have proven conclusively that schemes involving only the formation of free intermediate Compounds 2, 3, and 4 do not provide acceptable explanations for the observable rate data. A significant portion of the experimentally produced pregnenolone (from cholesterol) clearly cannot be accounted for by the sum of several reactions involving 2, 3, and 4 as isolable intermediates. Furthermore, Van Lier and Smith (10) recently have considered another oxygenated derivative, 20α-hydroperoxycholesterol, 5, as a possible intermediate. They have reported that incubation of this compound with an adrenal mitochondrial fraction containing cytochrome P-450 under an atmosphere of 100% nitrogen and in the absence of NADPH results in the formation of the glycol, 4. Supposedly, the product arises via an enzyme-catalyzed intramolecular rearrangement of the hydroperoxide to the glycol, with retention of both oxygen atoms from the peroxy group.

Two previous alternate proposals for the biosynthetic conversion of 1 into 6 include (a) the suggestion of Hall and Koritz (11) that Compounds 2 and 4 are formed as intermediates but remain tightly bound to the enzyme, never being released into the surrounding medium, and (b) the idea (8) that the steroid molecule 1 and O2 form a complex with the enzyme followed by the liberation of the side chain by some unspecified concerted process. Recently, some model, nonenzymic reactions have lent support to a third hypothesis which is considered in this paper. Simpson and Boyd (8) have already pointed out that this is an unlikely possibility. Moreover, Burstein et al. (9) have conducted an elaborate kinetic analysis of the pertinent reactions and have proven conclusively that schemes involving only the formation of free intermediate Compounds 2, 3, and 4 do not provide acceptable explanations for the observable rate data. A significant portion of the experimentally produced pregnenolone (from cholesterol) clearly cannot be accounted for by the sum of several reactions involving 2, 3, and 4 as isolable intermediates. Furthermore, Van Lier and Smith (10) recently have considered another oxygenated derivative, 20α-hydroperoxycholesterol, 5, as a possible intermediate. They have reported that incubation of this compound with an adrenal mitochondrial fraction containing cytochrome P-450 under an atmosphere of 100% nitrogen and in the absence of NADPH results in the formation of the glycol, 4. Supposedly, the product arises via an enzyme-catalyzed intramolecular rearrangement of the hydroperoxide to the glycol, with retention of both oxygen atoms from the peroxy group.

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Since the model experiment reported by Lieberman et al. (12) indicates that prior oxygenation at C-22 may not be necessary for the biosynthetic cleavage of the side chain, an analog of 20α-hydroxycholesterol, having no replaceable hydrogen atom on C-22, has been synthesized and tested as a biochemical precursor. It was hoped that such a trial might provide useful information concerning the mechanism of the cleavage reaction. Several analogs of 2 had been previously prepared (7, 16, 17), and these are compounds in which the isohexyl side chain of 20α-hydroxycholesterol, R-C20(CH3)(OH)-isohexyl (R = C19 steroidal fragment), was replaced by methyl, ethyl, isopropyl, isobutyl, or isoamyl substituents. With the exception of the C-20 methyl derivative, each of these is converted into pregnenolone or progesterone by adrenal preparations. As is evident, C-22 in these compounds is incompletely substituted, and, consequently, it is impossible to decide whether or not prior oxygenation at C-22 is required for the formation of pregnenolone. In the present study, the t-butyl analog 9, in which C-22 is fully alkylated, was used as a substrate in an in vitro experiment with a rabbit and in in vivo experiments with mitochondria from bovine adrenal glands.

**EXPERIMENTAL PROCEDURE**

All solvents were distilled prior to use. Partition column chromatography was performed as described by Siteri (18). Infrared spectra were determined in potassium bromide with a Perkin Elmer 221 infrared spectrophotometer. Melting points were determined on a Kofler block and are corrected. Nuclear magnetic resonance spectra were recorded with a Varian A-60 spectrometer. Mass spectra were obtained with an MS-9 mass spectrometer. Tracer [7α-3H]pregnenolone (specific activity 25 Ci per mmole) was purchased from New England Nuclear Corp. and was purified by Celite chromatography with System C-1 (Table I) where pregnenolone is eluted in hold-back volumes 7 to 9. (According to the manufacturer's specification, about 95 to 98% of the tritium is at C-7, and about 10% is at C-4.) The radiochemical homogeneity of the purified tracers was established by crystallization to constant specific activity with carrier pregnenolone. Radioactive samples were counted in a Packard model 3375 liquid scintillation spectrometer (35.5% efficiency for 4H) to a counting error of \( \pm 5\). T-butylithium (2.26 M in n-pentane) was purchased from Alpha Inorganics Inc. A custom analysis of this reagent by gas-liquid chromatography indicated the presence of isobutylithium (approximately 0.5%).

**TABLE I**

**Chromatography systems**

<table>
<thead>
<tr>
<th>Column-Celite partition system</th>
<th>C-1</th>
<th>C-2</th>
<th>T-1</th>
<th>T-2</th>
<th>T-3</th>
<th>T-4</th>
<th>T-5</th>
<th>T-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-1</td>
<td>Benzene-ethyl acetate (1:1)</td>
<td>Chloroform-methanol (19:1)</td>
<td>Benzene-ethyl acetate (4:1)</td>
<td>Benzene-ethyl acetate (9:1)</td>
<td>Methylene chloride-acetone (25:1)</td>
<td>Benzene-ethyl acetate (5:1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Preparation of (20R)-20-t-Butyl-5-pregnene-3ß,20-diol, 9**

Because the t-butyl analog 9 could not be prepared by means of a Grignard reagent, it was synthesized by condensing pregnenolone acetate with t-butylithium. Pregnenolone acetate (2 g, 5.00 mmole) in toluene (40 ml) was stirred under nitrogen in a Dry Ice-ethanol bath at \(-70^\circ\). T-Butylithium (30 ml, 2.26 M in n-pentane, 67.8 mmole) was added over a period of 4 hours. Water (300 ml) was added to decompose the reagent. After the mixture was warmed to room temperature, it was extracted with two 150-ml portions of ether. The ether extracts were dried over magnesium sulfate and evaporated to a colorless oil which was chromatographed on a Silica Gel H column (90 g) with an ethyl acetate-isooctane mixture (3:20). The products, in order of elution, were the following: Fraction I: (20R) 3ß-acetoxy-20-t-butyl-5-pregnene-20-ol (282 mg, 0.56 mmole, 10%): needles from methanol, m.p. 262-267°; infrared spectrum (KBr) 1720 cm\(^{-1}\) (acetate C=O) and 3490 cm\(^{-1}\) (20α-OH).

\[ \text{C}_{28}\text{H}_{45}O_2 \]

Calculated: C 77.84, H 10.65
Found: C 77.50, H 10.50

Fraction II: pregnenolone acetate (650 mg, 1.8 mmole, 33%), unchanged. Fraction III: (20R)-20-t-butyl-5-pregnene-3ß,20-diol, 9 (324 mg, 0.87 mmole, 10%): m.p. 262-267°; infrared spectrum (KBr) 1720 cm\(^{-1}\) (acetate C=O) and 3490 cm\(^{-1}\) (20α-OH).

Fraction IV: (20R)-20-t-butyl-5-pregnen-3ß,20-diol, 9 (232 mg, 0.56 mmole, 10%): needles from methanol, m.p. 262-267°; infrared spectrum (KBr) 1720 cm\(^{-1}\) (acetate C=O) and 3490 cm\(^{-1}\) (20α-OH).

**Preparation of (20R)-20-t-Butyl-3ß-3H]5-pregnene-3ß,20-diol, 9**

Purification of t-Butanol-t-Butanol (1 liter, A. R. grade, Amend Drug and Chemical Co., b.p. 81.5-83.0°) was heated at reflux temperature for 5 hours with potassium permanganate (10 g) and anhydrous potassium carbonate (5 g), and then filtered through a bed of Celite. The filtrate was distilled through a fractionating column, and 100-ml fractions were collected. Those fractions boiling at 82.3-82.4° (literature: b.p. 82.8°) were combined for the preparation of the chloride.

**t-Butyl Chloride—Pure t-butanol (420 ml) was shaken vigorously with concentrated HCl (1100 ml), and the mixture was allowed to stand at room temperature for 15 min. The lower, acidic layer was saturated with CaCl\(_2\) and the mixture was shaken. After the layers were allowed to separate, the upper, organic phase of t-butyl chloride was collected and washed successively with 5% NaHCO\(_3\) solution and water. The product was dried over CaCl\(_2\) and then distilled through a fractionating column. The fraction distilling at 50.5° (literature: b.p. 50.7°) was collected. It was redistilled from potassium carbonate before use.**

**t-Butylithium—Lithium metal (2 g, 0.29 mole) was melted in dry paraffin oil at 180°. Sodium metal (100 mg) and a few drops of oleic acid were added, and the mixture was shaken vigorously. Upon cooling, the liquid was decanted, and the fine lithium particles were washed free of oil with n-pentane and anhydrous ether. The metal was introduced with 50 ml of ether into a three-necked reaction flask equipped with a magnetic stirrer, a CaCl\(_2\) drying tube, and a nitrogen inlet. The mixture was cooled to \(-40^\circ\).**
butyllithium was used directly for the condensation with [3H]-pregnenolone acetate (2 X 10^6 cpm, 10 nmoles), in 1 ml of toluene. 0.1 ml of the ether solution was used as the initiator.) Following 3 hours of vigorous stirring, the ether solution containing t-butyl chloride (15 ml, 0.14 mole) was added dropwise with stirring. After the reaction had proceeded for 30 min, an excess of water was added to decompose the reagent was prepared from 4 g of magnesium (0.16 mole) in 100 ml of ether. t-Butyl chloride (15 ml, 0.14 mole) was added dropwise in 1 ml of 20% (w/v) aqueous Na2SO3 solution. The mixture was initiated by the addition of 0.1 ml of a solution of freshly prepared t-butyllithium in ether. When the mixture had been shaken for 30 min, an excess of water was added to decompose the reagent, and the aqueous mixture was extracted with ether. The organic solvent was evaporated with nitrogen, and the residue was partitioned between water and ether, neutralized with acetic acid, and the methanol was evaporated. The residue was partitioned between water and ether, and the ether-soluble fraction was subjected to chromatography. The residue was partitioned between water and ether, and the ether-soluble fraction was subjected to chromatography. The residue was partitioned between water and ether, and the ether-soluble fraction was subjected to chromatography.

**TABLE II**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Weight</th>
<th>Amount</th>
<th>Specific activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
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<td>10,040</td>
<td>10,770</td>
<td>4,030</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.994</td>
<td>10,450</td>
<td>10,510</td>
<td>3,350</td>
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<tr>
<td>Methanol</td>
<td>0.348</td>
<td>13,040</td>
<td>10,000</td>
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<tr>
<td>Methanol</td>
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<td>13,170</td>
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<td>Methanol</td>
<td>0.516</td>
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<td>Methanol</td>
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<td>42</td>
<td>117</td>
<td>23</td>
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</tr>
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<td>Benzene</td>
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<td>Benzene</td>
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<td>Benzene</td>
<td>0.408</td>
<td>332</td>
<td>702</td>
<td>251</td>
</tr>
</tbody>
</table>

* X-n = crystalline product from the n-th crystallization; ML-n = residue left in mother liquor from the n-th crystallization.

Conversion in vivo of (20R)-20-t-Butyl-5-pregnen-3β,20-diol, 9, into Pregnanediol

Experiment I—A female rabbit weighing 4 kg was injected intravenously with 3,250,000 cpm (0.17 nmole) of (20R)-20-t-butyl-[7α-3H]pregnene-3β,20-diol, 9, dissolved in 1 ml of 20% ethanol-0.09% NaCl solution. Within a few minutes, 10
units of adrenocorticotropic hormone gel (Organon) and 2000 units of chorionic gonadotropin (APL-Ayerst Laboratories) were administered subcutaneously. The urine (300 ml), collected over 2 days, was adjusted to pH 3 with 1 N HCl and filtered through glass wool. The acidified urine was diluted with water to 500 ml, and the steroid conjugates were absorbed on a XAD-2 column as described by Bradlow (19). The material extracted from the column with methanol was dissolved in 200 ml of 0.1 N sodium acetate buffer, pH 5, and Ketodase (1000 units per ml) was added. The solution was incubated at 40° for 72 hours. Eighteen milligrams of pregnenediol (56 umoles) were added as unlabeled carrier, and the incubation mixture was extracted three times with 250 ml of ether. The combined ether solutions were washed three times with 50-ml portions of water and evaporated to dryness. The residue, containing 100,000 cpm, was chromatographed on Celite with System C-2 where the carrier and a peak of radioactivity were both eluted in hold-back volume 4. The material in hold-back volume 4 was dissolved in CHCl₃ and the solution was poured onto a 5-g column of alumina (6% H₂O). The column was developed with 0.5% methanol in CHCl₃, and the fractions containing pregnenediol were combined and evaporated to dryness. The residue was recrystallized twice, and the specific activities of the products are listed in Table II. A portion of the material from the second crystallization was oxidized to 3S-pregnen-3,20-dione with Cr₂O₇. Pregnanediol was dissolved in 0.5 ml of acetic acid, and a solution of Cr₂O₇ (25 g, 0.25 mole, in 100 ml of 90% acetic acid) was added until the color of the oxidant persisted. A small excess of oxidant was added, and the solution was left to stand at room temperature for 30 min. The Cr₂O₇ was destroyed with 1 ml of methanol, and the reaction mixture was poured into water. A sodium hydroxide solution (5 N) was carefully added until the pH was adjusted to 7.4. Following centrifugation at 600 x g for 15 min, the supernatant was poured off and recentrifuged at 7000 x g for 20 min. The resulting mitochondrial pellet was suspended in 270 ml of the isolation medium and spun at 600 x g for 15 min; the supernatant was poured off and recentrifuged at 7000 x g for 20 min. The mitochondrial pellet was resuspended in the medium, and the suspension was centrifuged again for 20 min at 7000 x g. A portion of the resulting mitochondrial pellet was suspended in 2 ml of 0.1 M tris(hydroxymethyl)aminomethane-HCl buffer (Tris) (pH 7.4) and sonicated four times for 15-sec intervals with a 60-watt micro tip (Ultrasonic Inc., model W 1850). The temperature during sonication was maintained between 0 and 4° by keeping the sample in an ice bath and allowing 30-sec intervals between each sonication.

Isolation of Bovine Adrenal Mitochondria

Fresh bovine adrenocortical tissue (100 g defatted and demedullated) was suspended at 4° in 1000 ml of a medium which consisted of 0.25 M sucrose, 3 mM N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) (pH 7.4), 1 mM EDTA, and 0.05% bovine serum albumin. The mixture was then homogenized, and the pH was adjusted to 7.4 with 1 N KOH. Following centrifugation at 600 x g for 15 min, the supernatant was poured off and recentrifuged at 7000 x g for 20 min. The resulting mitochondrial pellet was suspended in 270 ml of the isolation medium and spun at 600 x g for 15 min; the supernatant was poured off and recentrifuged at 7000 x g for 20 min. The mitochondrial pellet was resuspended in the medium, and the suspension was centrifuged again for 20 min at 7000 x g. A portion of the resulting mitochondrial pellet was suspended in 2 ml of 0.1 M tris(hydroxymethyl)aminomethane-HCl buffer (Tris) (pH 7.4) and sonicated four times for 15-sec intervals with a 60-watt micro tip (Ultrasonic Inc., model W 1850). The temperature during sonication was maintained between 0 and 4° by keeping the sample in an ice bath and allowing 30-sec intervals between each sonication.

Incubation Experiments

Experiment II—(20R)-20-t-Butyl-[7α-3H]-pregnen-3β,20-diol, 9, (5.36 x 10⁵ cpm, 0.3 nmole) was added to two separate incubation tubes (a and b). The solvent was evaporated, and the residue was dissolved in 0.1 ml of acetone containing 10 μg of Tween 80. The acetone was evaporated, and 0.5 ml of the sonicated mitochondrial suspension (equivalent to 2.5 g of tissue) was placed in tube a. The other tube (b) served as a control and received mitochondria that had been heated at 100° for 5 min. To each tube, the following were introduced: 0.2 ml of 0.1 M Tris-HCl buffer, pH 7.4, 0.1 ml of 0.1 M CaCl₂, 0.1 ml of a NADPH-generating system (1.9 mg (2.5 μmoles) NADP, 6.25 mg (20.6 μmoles) glucose 6-phosphate, 0.5 unit of glucose 6-phosphate dehydrogenase (Sigma)) in 0.1 ml of phosphate buffer, pH 7.4.

Experiment III—(20R)-20-t-Butyl-[7α-3H]-pregnen-3β,20-diol, 9, (2.7 x 10⁵ cpm, 0.1 nmole) was added to three tubes (a, b, and c). 20α-[7α-3H]Hydroxycholesterol (4.71 x 10⁴ cpm, 1.8 μmoles) (the preparation and purification of which has been previously described (4)) was put into a fourth tube (d). One milliliter of the sonicated mitochondrial suspension (8.75 mg of protein, equivalent to 6 g of bovine tissue) was added to tubes a, b, and d while tube c, the control, received boiled mitochondria. To each tube was added twice the volume of the solutions of the above-mentioned cofactors.

The samples described above were shaken in air for 30 min at 37° after which carrier pregnenolone was introduced. The contents of each tube were extracted twice with 5 volumes of ether, and the extracts were evaporated and chromatographed on 66 g of Celite with System C-1. The fractions containing pregnenolone (hold-back volumes 7 to 9) were pooled, evaporated, and the resolved carrier was characterized in the following manner.

Experiment IV—The carrier pregnenolone (20.5 mg, 65 μmoles), after isolation from System C-1, was twice crystallized from methanol. The specific activities of the crystals and of the residues remaining in the mother liquids are given in Table II. The product of the second crystallization was acetate washed with acetic anhydride in pyridine, and the specific activities of the samples obtained by two successive crystallizations from methanol were determined. The specific activity of the twice recrystallized pregnenolone acetate was 965 cpm per μmole (X-2 in Table II), which corresponds to a total of 0.2500 cpm recovered as pregnenolone or to a 1% yield.

Experiment V—The radiochemical homogeneity of the pregnenolone (20.4 mg, 65 μmoles added) recovered from the tube in
which 9 was incubated with heat-treated mitochondria was not established. The sample obtained from the Celite chromatogram was recrystallized from methanol. Its specific activity was 23 cpmp per pmole. After a second recrystallization from the same solvent, the value decreased to 17 cpmp per pmole.

Experiment IIIa—After chromatography on Celite, System C-1, those fractions containing carrier pregnenolone (17.5 mg, 55 pmole) were rechromatographed successively in thin layer chromatography Systems T-1 and T-2. The product recovered from thin layer chromatography T-2 was recrystallized twice. The specific activities of the products and of the residues left in the mother liquors are listed in Table II. The product from the second crystallization was acetylated, and the pregnenolone acetate was crystallized twice. The specific activities of both the crystals and the residues left in the mother liquors are given in Table II. The specific activity of IIIa-X-2 (190 cpmp per pmole) corresponds to a total recovery of 10,500 cpmp as pregnenolone, a yield of 0.46%.

Experiment IIIb—Pregnenolone (16.3 mg, 52 pmole, introduced as carrier) was reisolated by chromatography with System C-1. The product was oxidized to progesterone by the Oppenauer procedure. It was dissolved in 10 ml of toluene and 3.3 ml of cyclohexanone, and the solution was heated to boiling in order to remove traces of water by azeotropic distillation. Aluminum isopropoxide (20 mg, 98 pmole) was then added, and heating was continued for 90 min. The reaction mixture was cooled and diluted with ether. After removal of solids, the solution was washed with 1 N HCl (50 ml), 1 N NaOH (50 ml), and with water until neutral. The organic extract was dried over Na2SO4 and evaporated to dryness in a flash evaporator. The residue was chromatographed on Celite with System C-1. Progesterone (11.3 mg, 36 pmole, 242 cpmp per pmole) was found in the third hold-back volume (unreacted pregnenolone was found in hold-back volumes 7 to 9) and was rechromatographed in thin layer chromatography System T-1 where it migrated with an Rf of 0.5. The resulting sample of progesterone (specific activity 233 cpmp per pmole) was recovered from the thin layer chromatography plate by extraction with chloroform and was reduced enzymically to 20β-hydroxy-4-pregnen-3-one. The sample of progesterone (10.9 mg, 35 pmole) was dissolved in 2 ml of ethanol and added slowly to 40 ml of a solution (0.1 M Tris-HCl, pH 7.4, 10-4 M EDTA) containing 1 mg of 20β hydroxy steroid dehydrogenase (Streptomyces hydrogenans-Sigma type II) and 100 μg of NADH. The mixture was gently agitated for 2 hours at room temperature and then extracted twice with 100 ml portions of ether. The ether solution was washed twice with 50 ml of H2O, dried over anhydrous Na2SO4, evaporated, and the residue was chromatographed in System T-1. The product, 20β-hydroxy-4-pregnen-3-one (2 mg, 77 pmole), migrating with an Rf of 0.4 (progesterone Rf = 0.5), was recovered from the plate and acetylated overnight at room temperature in 0.5 ml of pyridine and 0.25 ml of acetic anhydride. The reaction mixture was diluted with water and extracted into 100 ml of ether. The ether extract was washed with 50 ml of 0.1 N HCl, with water, dried over Na2SO4, and evaporated to dryness. The residue was chromatographed by thin layer chromatography in System T-3 (Rf = 0.4). The isolated 20β-acetoxy-4-pregnen-3-one (7.4 mg, 21 pmole) was extracted from silica gel with chloroform and recrystallized once from methanol and twice from acetone-petroleum ether. The specific activities of the products and of the residues in the mother liquors are listed in Table II. From the specific activity of IIIb-X-3 (251 cpmp per pmole), the yield of pregnenolone isolated from 9 was calculated to be 0.6%. The Product IIIb-X-3 (m.p. 168-170°) was shown to be identical (m.p., infrared spectrum, ultraviolet absorption at 240 nm, and chromatographic mobility) with a sample of 20β-acetoxy 4-pregnen-3-one prepared by acetylation of an authentic sample of 20β-hydroxy-4-pregnen-3-one (obtained from Upjohn Co.).

Experiment IIIc—After chromatography in System C-1, the fractions containing carrier pregnenolone (15.6 mg, 49 pmole, added) were pooled and rechromatographed in System T-1. Pregnenolone, isolated after the incubation of the tritiated analog 9 with the heat denatured enzyme, had an apparent specific activity of 4 cpmp per pmole which corresponds to a conversion of less than 200 cpmp (<0.009%). No further effort was made to establish whether or not the radioactivity was associated with the carrier.

Experiment IIId—Pregnenolone (16.0 mg, 51 pmole, added), following incubation of 20α-[7α-3H]hydroxycholesterol, was isolated by chromatography with System C-1 and was recrystallized from methanol to constant specific activity (2885 cpmp per pmole). The number of counts per min recovered as pregnenolone was 120,500 cpmp which corresponds to a yield of 26%.

Location of Tritium in Pregnenolone (6) Resulting from Incubation of t-Butyl Analog 9 with Bovine Adrenal Mitochondria

In order to strengthen the conclusion that 9 was directly transformed into 6 in Experiments II and III, it was desirable to eliminate the possibility, however unlikely, that the isotope in the pregnenolone isolated following the in vitro metabolism of 9 was no longer attached to C-7, as it is in the Precursor 9, but was located at some other position in the molecule, having been introduced by some indirect process.

That the tritium present in biosynthesized pregnenolone remains at C-7 following incubation of the t-butyl analog 9 was shown by the almost complete loss of radioactivity from the product when it was converted into 3β-acetoxy-5-pregnen-7,20-dione (7-ketopregnenolone acetate). Fractions containing tritiated pregnenolone isolated from several incubations of 9 with bovine adrenal mitochondria were combined. Each of these fractions contained carrier pregnenolone and previously had been shown to be radiochemically homogeneous. After further dilution with unlabeled pregnenolone, these pooled fractions were acetylated with acetic anhydride in pyridine, and the acetate was purified by chromatography in System T-3. The product was eluted from two thin layer plates with chloroform, and, after evaporation of the solvent, the residue was crystallized from aqueous methanol. The crystalline pregnenolone acetate (27 mg, 75 pmole) had a specific activity of 311 cpmp per mg. It was dissolved in 0.35 ml of acetic acid, the solution was heated to 53-75°, and 25 mg (230 pmole) of crystalline CsO2 was added in five nearly equal portions over a period of 2 hours. The solution was kept at 53-55° for an additional 2 hours after which the excess oxidant was destroyed with 0.1 ml of methanol. The mixture was added to 20 ml of 1 N NaOH, and the organic material was extracted with 50 ml of ether. The ether extract was washed with 10 ml of water, dried over Na2SO4, and evaporated to dryness. Using System C-1 (with hexane substituted for isooctane), the residue was chromatographed on Celite, and the 7-ketopregnenolone acetate was found in hold-back volume 4 to 5. These fractions were combined and evaporated to dryness. The resi-
exhibits a characteristic broad band between 3600 and 3400 cm⁻¹ 2

20α-hydroxycholesterol while, in the same region, the 20β isomer is common to the infrared spectrum (KBr) of the 3β-acetate of pregnenolone from which it was derived. The specific activity of the 7-keto compound was found to be 23 cpm per mg, less than 8% of the specific activity of the pregnenolone acetate. Three experimental observations collectively were used to assign the α configuration to the C-20 hydroxy group. First, the positions of the nuclear magnetic resonance, infrared, and mass spectral data are entirely consistent with the predicted structure of 9, as is the elemental analysis of its acetate. Third, it has been shown that Grignard reagents normally add to pregnenolone to give, almost exclusively, 20α-hydroxylated products (21). Hence, it is presumed that the C-20 hydroxy group of 9 has the α configuration, although this assignment cannot be considered to be rigorously proven. (In the notation of Cahn, Ingold, and Prelog (22), the 20α-hydroxy group in 9 has the R configuration while the 20β-hydroxy groups in 2 and in 4 have the S configuration.)

Due to the limits of detection imposed by nuclear magnetic resonance and infrared spectral techniques, a few per cent of the 20β epimer, if present in a sample of 9, would not be noticed. Thus, nuclear magnetic resonance and infrared data cannot be used as criteria for the stereochemical purity of 9. However, it is likely that the exhaustive, highly specific, and selective chromatographic procedures to which the Tracer 9 was subjected prior to its use in the biological experiments would have separated the 20α and 20β epimers. Nevertheless, since such a separation was not experimentally verified, there exists the possibility that the t-butyl analog used in the biological experiments contained a few per cent of the 20β epimer. In either case, the general mechanistic interpretation (see below) of the observed conversion of 9 into 6 would remain the same.

Following the intravenous administration of tritiated 9 to a rabbit, radioactive pregnenadiol, 10, was isolated from the animal's urine in 0.04% yield. For comparison, tritiated pregnenolone, 6, was administered intravenously to another rabbit, and the urinary diol 10 was isolated in 0.5% yield. When incubated with sonicated adrenal mitochondria for 30 min, tritiated 9 was converted into tritiated pregnenolone, 6, in 0.5 to 1.2% yield (Scheme I). When 20α-hydroxycholesterol, 2, was incubated under the same conditions, the yield of pregnenolone derived from it was 26%.

As might be expected, because of the bulky side chain present in 9, it is a poor precursor of pregnenolone when compared with 20α-hydroxycholesterol, 2, or even with cholesterol, 1. This was not nearly so evident in the in vivo experiment as it was in the in vitro experiments with adrenal mitochondria. However, that 9 was oxidized by the side chain cleavage enzyme system indicates that a significant part of the specificity of the enzyme for the substrate is probably directed toward the Δ₅,3β-hydroxy portion of the C₁₉ steroidal nucleus (23), a common feature of 1, 2, and 9. The poor yield of pregnenolone from 9 requires a close examination of the experimental conditions, particularly with regard to the in vitro incubations, to assure lack of interference by undesirable substances. It is apparent that contamination of the substrate with an isomer not fully substituted at C-22 would be unacceptable since it has been shown (7) that analogs in which C-22 bears a hydrogen substituent can serve as precursors of pregnenolone. Commercial preparations of t-butyl chloride, which boiled at 50.5°C (literature b.p. 50.7°C), was obtained. The purified t-butyl alcohol of proper boiling point was used to prepare t-butyl chloride. This halide was made by treatment of the alcohol with concentrated hydrochloric acid and purified by fractional distillation whereby a sample of the chloride, which boiled at 50.5°C (literature b.p. 50.7°C), was obtained.

Scheme I. Metabolism of the t-butyl analog 9 in vivo and in vitro.
of t-butyl chloride. These precautions would seem to preclude the possibility that the experimental results might be explained by the presence of a minor impurity in 9. Further, it should be noted that, unless the efficiency of its conversion were extraordinarily high, such a contaminant would have had to be present in considerable quantity to account for the observed yields of pregnenolone.

Since the starting material, \([7\alpha^{-3}H]\)pregnenolone, was a potential contaminant of the tracer 9, any chance of its presence was carefully eliminated by "wash-out" chromatograms. Only tracer purified in this way was used for the biological experiments. In order to eliminate the possibility that the \([\Pi]\) present in the pregnenolone isolated following the in vitro metabolism of 9 had been introduced into the product by indirect means, an effort was made to determine whether the radioisotope was present in the product at the same position, C-7, as it had been in the starting material, 9. To this end, \([\Pi]\)pregnenolone, obtained from several in vitro incubations of 9, was converted into 7-ketopregnenolone acetate which contained almost no tritium. Indirect transfer of tritium, if it had occurred, would probably not have resulted in labeling of the product, 6, at C-7, and thus it is reasonable to believe that the tritium at C-7 was retained during the conversion of 9 into 6. Further proof that this conversion was not artifactual was shown by the fact that the control incubation of untraded 9 with adrenal tissue, previously boiled, did not result in the isolation of radioactive pregnenolone.

In all tracer experiments of this type, the radiochemical homogeneity of both the starting material and the products (24) must be rigorously proven. Evidence for the radiochemical purity of the t-butyl analog 9 is presented in Table II. The pregnenolone isolated from the in vitro incubations was examined by two procedures leading to different derivatives. In each case, radiochemical homogeneity was established by exhaustive chromatography and crystallization. In one instance, pregnenolone and its acetate were the final products. In the other, pregnenolone was dehydrogenated, and the resulting progesterone was, in turn, reduced by the specific enzyme 20\(\alpha\)-hydroxysteroid dehydrogenase to 20\(\alpha\)-hydroxy-4- pregnen-3-one. The latter was characterized as its acetate. The pregnenediol isolated from the in vivo experiment was characterized as such and as its diketone, pregnandione.

Insofar as they bear on the discussion of the biosynthetic pathways and reaction mechanisms discussed in this paper, though, the small yields of pregnenolone from 9 are of little consequence because they cannot be evaluated in isolation. At best, the yields can only be judged in comparison with the results obtained with a presumed natural precursor, pregnenolone. When administered intravenously into a rabbit, even this natural precursor was converted into urinary pregnenolone in a yield of only 0.5\%, the bulk having been diverted into other unaccounted for fates. For the artificial analog, 9, to be converted into and excreted as pregnenolone, it must first have been transported by the blood to the few select organs that are capable of transforming it into pregnenolone. In the course of this transport, the bulk of the unnatural compound undoubtedly is catabolized irreversibly into many unstable metabolites. When 9 arrives at the proper organ or organs, it unquestionably serves as a much poorer substrate for whatever natural enzyme system is ordinarily available for the formation of pregnenolone from cholesterol. Pregnenolone formed from 9, or more likely the progesterone produced from it, would then have to be transported to the liver where the conjugated form of pregnenolone (probably the glucuronide) would be formed in preparation for excretion into the urine. In our view, the efficiency of the over-all in vivo process, t-butyl analog → urinary pregnenolone, is of little importance. On the other hand, the mechanisms by which the conversion 9 → 6 occurs does deserve consideration, for the sake of our understanding of the intimate details of the natural transformation of cholesterol into pregnenolone.

**Possible Intermediates in Process:** 9 → 6—With the demonstration that the t-butyl analog 9 can serve as a precursor of pregnenolone, mechanisms for this reaction may be envisaged. It is very unlikely that C-22 is oxygenated prior to the side chain cleavage of the t-butyl analog 9. For this to occur, demethylation of the t-butyl group would first be necessary. Demethylation of steroids, as in the conversion of lanosterol into cholesterol (26), usually proceeds by hydroxylation of the methyl group followed by stepwise oxidation to the carboxylic acid (\(-\text{CH}_3 \rightarrow -\text{CH}_2\text{OH} \rightarrow -\text{CHO} \rightarrow -\text{COOH}\) and ionic decarboxylation. Facile loss of carbon dioxide is made possible by the presence of a \(\beta\)-carbonyl group or of a \(\beta,\gamma\)-carbon-carbon double bond. Such suitably located functional groups are not present in 9. Alternatively, oxidative decarboxylation, whereby electron removal from the carboxyl group results in a highly CO-substituted, aliphatic carboxylic radical (\(\text{RC}O_2^- \rightarrow \text{RCO}_2^- \rightarrow \text{R}^- + \text{CO}_2\)), may be feasible, but there is no reason to favor the lengthy, over-all process of demethylation and subsequent oxygenation over a simple, direct radical side chain cleavage mechanism such as that suggested in Scheme II. Thus, it is most probable that demethylation (and therefore oxygenation) of C-22 in 9 does not occur prior to side chain cleavage.

Two general mechanisms, one radical and one ionic in nature, for the conversion of the t-butyl analog 9 into pregnenolone are drawn in Scheme II. Although it is simpler to represent the reaction by these two opposite, mechanistic extremes, the actual cleavage process may be some hybrid of these two mechanisms.

1. "The distinction between pathways and mechanisms is one between the determination of isolable intermediates and the elucidation of the transition states for particular chemical processes; the two concepts overlap somewhat where unstable intermediates must be postulated for a reaction" (25).
SCHEME III. Hypothetical radical pathways for the conversion of cholesterol into pregnenolone. For convenience, the transient intermediates are depicted as steroidal radicals but, as emphasized in the text, these reactive species are probably complexed with metalloenzymes, and their true structures cannot be defined so simply. This scheme is used only to suggest oxygenation patterns and reaction pathways, not detailed mechanisms and structures. Note—Compound 4 may also be converted into the intermediate shown here as the diradical.

Hydrogen atom abstraction to form a tertiary alkoxy radical (Path A) would be followed by fragmentation to pregnenolone via β scission, a well known behavior of alkoxy radicals (27). The resultant t-butyl fragment would then be available for conversion into C19 products by another concerted oxidation or reduction process, always under enzymic control so that radical species would not be released into the medium. It seems more reasonable to assume the formation of some sort of intermediate radical-enzyme (E) complex rather than the less likely production of “free” radical species.

One possible ionic mechanism illustrated by Path B involves the formation of an intermediate alkoxy-metal complex incorporating the trivalent metal ion (M(III)) from a metalloenzyme and an oxidant (written here as molecular oxygen).4 Many variations on this type of mechanism can be envisaged (28). The metal ion either may serve to transfer electrons to another oxidant or may itself serve as the electron acceptor. In the latter situation, its formal valence would change, while in the former case it would remain the same. Heterolytic cleavage of the carbon-carbon bond to give pregnenolone with concurrent 2-electron reduction of the oxidizing agent may be aided by concerted attack on the central carbon atom of the t-buty1 group. In this way, free carbonium ion intermediates might not be formed.

These greatly simplified radical and ionic schemes (Scheme II) each afford a rational mechanistic explanation for the oxidative cleavage of the t-buty1 analog 9. Therefore, it seems reasonable that side chain cleavage can occur without prior introduction of an oxygen function at C-22, although a mechanism in which carbon-carbon bond cleavage and C-22 oxidation (initially yielding t-butoxy species) are concerted must also be considered. Nevertheless, whatever the mechanism, cleavage of 9 would not involve a glycol-type intermediate.

Speculations Regarding Biosynthetic Pathways for Process: Cholesterol (I) → Pregnenolone (6)—Due to their plausibility, the simple mechanistic routes pictured in Scheme II for the oxidation of 9 can form grounds for speculation concerning the in vivo biosynthesis of pregnenolone from the natural precursor cholesterol. As explained earlier, the hydroxylated Compounds 2, 3, and 4 may not be the true intermediates in this process. Oxidation of cholesterol, catalyzed by a metalloenzyme (probably a type of mixed function oxidase), could produce short lived intermediate complexes; subsequent side reactions of these complexes would account for the formation of 2, 3, and 4. The reversibility of these competitive processes would explain the ability of 2, 3, and 4 to serve as precursors of pregnenolone. A schematic representation of these various pathways is given in Scheme III.

On the basis of current knowledge, it is not possible to decide whether the reactions involved in side chain cleavage of cholesterol have radical or ionic character. In fact, such a distinction may even be artificial; for example, if a metal ion may exist in more than one relatively stable valence state, and if a ligand may...
exist in more than one relatively stable oxidation state, then a complex of the metal and ligand may be considered as a resonance hybrid which cannot be represented faithfully by a single valence bond structure* (28). This concept is illustrated below: in 11a the metal ion complexed to the RO radical species has a valence of plus two, while in the metal-RO anion complex, 11b, the valence of the metal is plus three.

\[ R-O-M \rightleftharpoons R-O^{-}M^{2+} \]

Since the short lived intermediates proposed in Scheme III for the biosynthetic pathway from 1 to 6 are probably oxygenated steroidal species complexed in some way to a metalloenzyme, it is difficult to say whether they possess radical or ionic characteristics. Undoubtedly, the formation, behavior, and structure of such reactive intermediates are influenced by cofactors, by the medium, and, particularly, by the enzymes to which these species may be bound more or less tightly. Thus, only oxygenation patterns and reaction pathways may be suggested; detailed mechanisms and structures of the intermediate enzyme complexes cannot yet be implied.

It is not known whether initial oxygenation of cholesterol occurs at C-20 or at C-22, nor is it known whether at first a single oxygen atom or an entire oxygen molecule is transferred. As indicated in Scheme III, peroxygenated species, if formed, may be degraded to alkoxyl species, or they may rearrange to give a glycol-type transient. A C-20, C-22-dioxygenated species seems to be the most likely proximal intermediate preceding the final cleavage to pregnenolone and the C2 fragment. This is unlike the metabolism of the t-butyl analog 9 in which the formation of a glycol-type intermediate is not possible. In this latter situation, bond cleavage is favored by tetrasubstitution at C-22 since this carbon atom becomes a tertiary carbon center after cleavage. In cholesterol, on the other hand, C-22 is only disubstituted, and after side chain cleavage it formally becomes a primary carbon center. Introduction of oxygen at C-22 thus would seem to favor side chain cleavage of cholesterol since it would be unreasonable to assume the formation of a primary radical or cationic C2 hydrocarbon fragment. As previously stated, introduction of a second oxygen atom into a mono-oxygenated species may be concerted with cleavage of the C20 to C22 carbon-carbon bond, in which case the C2 and C4 products, pregnenolone and isooxoproide (20), would result without the intermediacy of a C27 cholesterol derivative having two adjacent oxygen functions in the side chain. Thus, while reasonable mechanisms can now be considered for the side chain cleavage of the t-butyl analog 9 by virtue of the unique limitations imposed by the compound’s structure, it is difficult to extrapolate these possibilities to the metabolism of cholesterol where even the overall pathway for this complex biosynthetic process remains speculative.

General nonradical mechanisms which are reasonable for mixed function oxidases seem to operate by transferring oxygen atoms via “oxenoid” mechanisms, analogous to the mechanisms of carbene reactions (28). Carbene-type intermediates are known to have either radical or electrophilic characteristics, depending upon the method of their generation. Another kind of enzyme model involves attaching a rigid, photochemically excitable molecule to the hydroxy group of a long chain alcohol or sterol in a manner such that upon photolysis, the excited moiety will abstract a hydrogen atom by relatively selective radical attack on a position remote from the site of attachment (31-33). One last approach to mimicking enzymic oxidations which has achieved a high degree of selectivity is the chlorination (oxidation) of alkylamines and other aliphatic compounds effected by the attack of sterically hindered nitrogen cation radicals (generated by the photolysis of N-chloroamines) in highly acidic media (34, 35).

Some additional, isolated chemical experiments deserve mention. VanLier and Smith (30) found that autoxidation of cholesterol in the presence of triplet oxygen yields, among other products, the 20α-hydroperoxide 5; in contrast, the reaction of 1 with singlet oxygen gives products derived mainly from attack on the Δ2 unsaturated system in Ring B (37). In the cleavage of olefins to carbonyl compounds by singlet oxygen, dioxetanes

\[ {\text{C}}-{\text{C}} \rightarrow {\text{O}}-\text{O} \]

are presumed to be intermediates (38). In the decomposition of dioxetanes into carbonyl products, the intermediary of a glycol biradical has been proposed (39). The significance of these observations in relation to the biochemical side chain cleavage of cholesterol remains to be elucidated.

Rational schemes for the biosynthetic conversion of cholesterol into pregnenolone which are alternatives to previously proposed routes have been advanced in this paper. They assume the participation of intermediate, transient, reactive species possessing apparent radical or ionic character. However, neither direct nor even circumstantial evidence favoring radical or nonradical mechanisms is easily obtained. Nevertheless, the schemes proposed herein offer attractive, alternative explanations for the data so far gathered and provide a hypothesis that lends itself to further experimental examination.

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