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Studies on the Biosynthetic Conversion of Cholesterol into Pregnenolone

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

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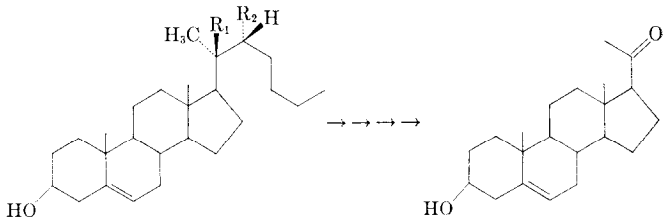
SUMMARY

(20R)-20-*t*-Butyl-5-pregnene-3β,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] **1**, is generally considered to be converted into pregnenolone, **6**, a precursor of all steroid hormones, by synthetic routes involving side chain-hydroxylated C_{27} 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



1 $R_1 = R_2 = H$
2 $R_1 = OH, R_2 = H$
3 $R_1 = H, R_2 = OH$
4 $R_1 = R_2 = OH$
5 $R_1 = OOH, R_2 = H$

6

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 C_{21} steroid at a rate faster than that of cholesterol. Further evidence bearing on their intermediacy 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 C_{27} sterol is converted into the C_{21} 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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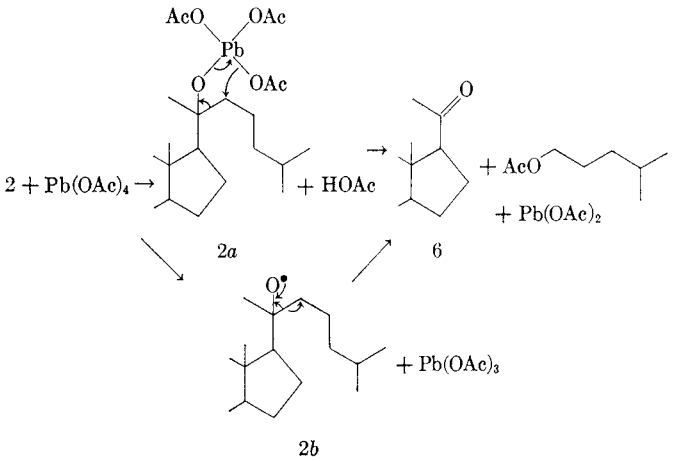
[1] Systematic names for steroids (1) given trivial names in the text are: **1**, 5-cholesten-3β-ol; **2**, 5-cholestene-3β,20α-diol; **3**, (22R)-5-cholestene-3β,22-diol; **4**, (22R)-5-cholestene-3β,20α,22-triol; **5**, 20α-hydroperoxy-5-cholesten-3β-ol; **6**, 3β-hydroxy-5-pregnen-20-one; **7**, 21-hydroxy-4-pregnene-3,20-dione; **8**, 4,16-androstadien-3-one; **9**, 24-Nor-22,22-dimethyl-5-cholene-3β,20α-diol; **10**, 5β-pregnane-3α,20α-diol; pregnanedione, 5β-pregnane-3,20-dione; progesterone, 4-pregnene-3,20-dione. *Note*: In the text, Compound **9** will be referred to as (20R)-20-*t*-butyl-5-pregnene-3β,20-diol to emphasize its structural analogy to Compound **2** and its other analogs.

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 O_2 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.

When 20α-hydroxycholesterol, **2,** is treated with lead tetraacetate, pregnenolone, **6,** is formed in good yield (12). The reaction sequence can be drawn as follows (Scheme 1).

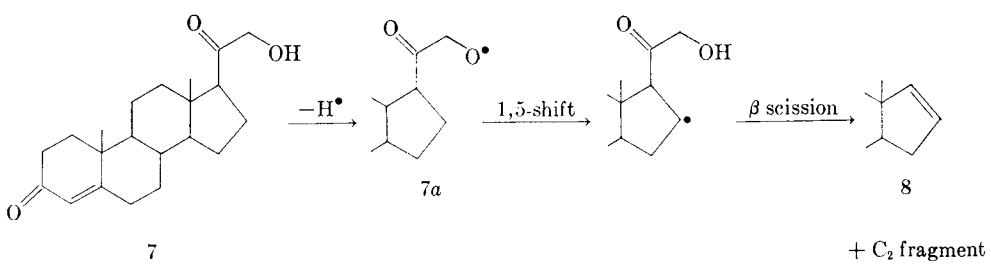


SCHEME 1

At the time of the original experiment, it was thought that formation of the intermediate alkoxy-lead Compound **2a** would be followed by its homolytic cleavage to the alkoxy radical **2b**. Then, β scission of **2b** would yield pregnenolone as shown (13). More recent evidence now favors a heterolytic pathway whereby **2a** may decompose in the concerted fashion indicated with resultant 2-electron oxidation of **2** to **6** and reduction of Pb(IV) to Pb(II) (14).

Treatment of deoxycorticosterone, **7,** with hydrogen-abstracting agents formed by the *in situ* decomposition of cumene hydroperoxide, *t*-butyl hydroperoxide, or phenylazotriphenylmethane (or photolysis of the C-21 nitrite of **7**) leads to the formation of the C_{19}-Δ^{16} compound, androst-4,16-dien-3-one, **8** (15). This transformation can be duplicated by *in vitro* incubation of the C_{21} corticosteroid **7** with an homogenate of boar testis tissue. These findings are best explained by a mechanism involving intermediates that behave as free radicals (Scheme 2).

While these two models do not necessarily reflect the mechanisms of the corresponding biosynthetic reactions, they do show the feasibility of the chemical processes and the reasonability of considering that suitably modified mechanisms of these types may operate in enzymic transformations. For example, it can be imagined that intermediates analogous in chemical behavior (if not in structure) to **2a, 2b,** or **7a** could be formed directly during the respective *in vivo* oxygenations of cholesterol (at C-20) and progesterone (at C-21) without the necessity of prior formation of **2** or **7**. In this view, then, Compounds **2** and **7** would not be true intermediates on the corresponding biosynthetic pathways to **6** and **8**; rather, they would be by-products resulting from competitive processes, possibly resembling such chemical reactions as the hydrolysis of **2a** or hydrogen atom abstraction by the radicals **2b** or **7a,** respectively. Schematically, according to this argument, the biosynthetic pathway to pregnenolone might be represented as **1** → **MC** → **6** and not as **1** → **2** → **MC** → **6** (where **MC** represents some metal complex which is the natural counterpart of **2a**); similarly, the pathway to the Δ^{16} metabolite might be written as: progesterone → **AR** → **8** and not as progesterone → **7** → **AR** → **8** (where **AR** represents some intermediate which is the natural counterpart of the alkoxy radical **7a**). Biogenesis of **2** and **7** then would follow the routes **1** → **MC** ⇆ **2** and progesterone → **AR** ⇆ **7**, respectively. By extension of this reasoning, Compounds **2, 3,** and **4** would not be intermediates on the direct biological route from cholesterol to pregnenolone. Instead, they would result from as yet undefined reversible transformations of the true intermediates. However, the presence of **2, 3,** and **4** in adrenal extracts suggests that the true intermediates, be they radical or ionic in character, are, possibly, oxygenated species bound in some fashion to a metalloenzyme. Attempts to secure further support for these new ideas are described in the present paper.



SCHEME 2

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 biological 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-$C_{20}(CH_3)$(OH)-isohexyl (R = C_{19} 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 vivo* experiment with a rabbit and in *in vitro* experiments with mitochondria from bovine adrenal glands.

EXPERIMENTAL PROCEDURE

All solvents were distilled prior to use. Partition column chromatography was performed as described by Siiteri (18). Infrared spectra were determined in potassium bromide with a Perkin Elmer 221 infrared spectrophotometer. Melting points were determined on a Köfler 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-^{3}H]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 85 to 90% 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 ^{3}H) to a counting error of $< \pm 5\%$. *t*-Butyllithium (2.26 M in *n*-pentane) was purchased from Alfa Inorganics Inc. A custom analysis of this reagent by gas-liquid chromatography indicted the presence of isobutyllithium (approximately 0.5%).

TABLE I

Chromatography systems

C = Column-Celite partition system: 0.7 ml (C-1) or 0.5 ml (C-2) of stationary phase per g of Celite; T = thin layer-Silica Gel GF: visualization-3-keto-Δ^4-steroids, ultraviolet light; 3β-hydroxy-Δ^5-steroids, Rhodamine G spray (0.02% in MeOH) followed by ultraviolet light.

C-1	Isooctane-methanol-water (10:9:1)
C-2	Isooctane-ethyl acetate-methanol-water (15:5:9:1)
T-1	Benzene-ethyl acetate (1:1)
T-2	Chloroform-methanol (19:1)
T-3	Benzene-ethyl acetate (4:1)
T-4	Benzene-ethyl acetate (9:1)
T-5	Methylene chloride-acetone (25:1)
T-6	Benzene-ethyl acetate (3:1)

Preparation of (20R)-20-t-Butyl-5-pregnene-3β,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*-butyllithium. Pregnenolone acetate (2 g, 5.6 mmoles) in toluene (40 ml) was stirred under nitrogen in a Dry Ice-ethanol bath at −70°. *t*-Butyllithium (30 ml, 2.26 M in *n*-pentane, 67.8 mmoles) 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 (60 g) with an ethyl acetate-isooctane mixture (3:20). The products, in order of elution, were the following: Fraction I: (20R)-3β-acetoxy-20-*t*-butyl-5-pregnen-20-ol (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).

$C_{27}H_{44}O_3$

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

Fraction II: pregnenolone acetate (650 mg, 1.8 mmoles, 33%), unchanged. Fraction III: (20R)-20-*t*-butyl-5-pregnene-3β,20-diol, **9** (324 mg, 0.87 mmole, 10%): m.p. 204–206.5°; nuclear magnetic resonance ($CDCl_3$, tetramethylsilane) δ 5.40 (m, 1, H-6), 3.50 (m, 1, H-3), 1.33 (s, 3, C-21 methyl), 1.00 (s, 3, C-19 methyl), 0.92 (s, 9, *t*-butyl), and 0.90 ppm (s, 3, C-18 methyl); mass spectrum (70 ev) *m/e* 374(parent), 373(M-H), 359(M-CH_3), 356(M-H_2O), 341(M-CH_3-H_2O), 317(M-C_4H_9), 299(M-C_4H_9-H_2O). When a sample of Fraction III was treated with acetic anhydride in pyridine, an acetate with a melting point and an ir spectrum identical with those of the acetate from Fraction I was obtained.

Preparation of (20R)-20-t-Butyl-[7α-^{3}H]5-pregnene-3β,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-Butyllithium—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°

with a Dry Ice-ethanol bath. Purified *t*-butyl chloride (10 ml, 0.092 mole) was then added in 50 ml of ether, and the reaction was initiated by the addition of 0.1 ml of a solution of freshly prepared *t*-butylmagnesium chloride in ether. (The Grignard 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 with stirring. After the reaction had proceeded for 30 min, 0.1 ml of the ether solution was used as the initiator.) Following 3 hours of vigorous stirring, the ether solution containing *t*-butyllithium was used directly for the condensation with [^{3}H]-pregnenolone acetate.

(20R)-20-t-Butyl-[7α-^{3}H]5-pregnene-3β,20-diol, 9—[7α-^{3}H]-Pregnenolone acetate (2×10^8 cpm, 10 nmoles), in 1 ml of toluene at −40°, was treated with 1 ml of the freshly prepared solution of *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 hydrolyzed at 60° for 1 hour in 5 ml of methanol containing 0.1 ml of 10% (w/v) aqueous Na_2CO_3 solution. The mixture was 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 on 33 g of Celite with System C-1. The Product **9** was eluted as a symmetrical peak (1.35×10^8 cpm, 7 nmoles) in hold-back volumes 3 to 4. Unreacted [7α-^{3}H]pregnenolone appeared in hold-back volumes 7 to 9. To remove traces of [^{3}H]pregnenolone from **9**, unlabeled pregnenolone was added to the fractions containing **9**, and the mixture was rechromatographed with System C-1. Insignificant amounts of tritium were associated with the material eluted in hold-back volumes 7 to 9. After an additional chromatographic analysis of **9** on Celite with System C-1, the radiochemical purity of the Tracer **9** was confirmed by crystallization with carrier (Table II). Another effort was made to determine whether the Product **9** was contaminated with the starting material. Fractions (hold-back volumes 7 to 9) from the last chromatogram in which tritiated pregnenolone would have appeared, if it were present, were diluted with unlabeled pregnenolone. The mixture was recrystallized from methanol, and the crystalline pregnenolone was found to be devoid of radioactivity. Just prior to use in the metabolic experiments, the tracer sample of **9** was rechromatographed employing System C-1 and then System T-1.

Conversion in vivo of (20R)-20-t-Butyl-5-pregnene-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-^{3}H]5-pregnene-3β,20-diol, **9**, dissolved in 1 ml of 20% ethanol-80% 0.09% NaCl solution. Within a few minutes, 10

TABLE II
Crystallization data

Experiment number	Sample	Fraction[a]	Solvent	Weight	Amount	Specific activity	Specific activity
				mg	*cpm*	*cpm/mg*	*cpm/μmole*
	(20R)-20-*t*-Butyl-5-pregnene-3β,20-diol	X-1	Methanol	0.932	10,040	10,770	4,030
	(20R)-20-*t*-Butyl-5-pregnene-3β,20-diol	X-2	Methanol	0.994	10,450	10,510	3,930
	(20R)-20-*t*-Butyl-5-pregnene-3β,20-diol	ML-1		1.348	14,290	10,600	3,960
	(20R)-20-*t*-Butyl-5-pregnene-3β,20-diol	ML-2		1.249	13,170	10,540	3,940
I	5β-Pregnane-3α,20α-diol	X-1	Methanol-ether	0.516	38	73	23
	5β-Pregnane-3α,20α-diol	X-2	Ether-petroleum ether	0.597	42	71	23
	5β-Pregnane-3,20-dione	X-1	Acetone-petroleum ether	0.329	25	74	24
IIa	Pregnenolone	X-1	Methanol	1.002	3,190	3,180	1,010
	Pregnenolone	X-2	Methanol	0.948	2,940	3,100	979
	Pregnenolone	ML-2		0.991	3,200	3,230	1,020
	Pregnenolone acetate	X-1	Methanol	0.810	2,210	2,730	977
	Pregnenolone acetate	X-2	Methanol	0.896	2,420	2,700	965
IIIa	Pregnenolone	X-1	Benzene	0.233	151	648	205
	Pregnenolone	X-2	Benzene-heptane	0.479	290	606	191
	Pregnenolone	ML-1		0.610	416	682	215
	Pregnenolone	ML-2		0.360	225	624	197
IIIa	Pregnenolone acetate	X-1	Heptane	0.725	375	518	185
	Pregnenolone acetate	X-2	Methanol	0.255	136	531	190
	Pregnenolone acetate	ML-1		0.630	334	531	190
	Pregnenolone acetate	ML-2		0.540	281	521	187
IIIb	20β-Acetoxy-4-pregnen-3-one	X-1	Methanol	0.503	346	687	246
	20β-Acetoxy-4-pregnen-3-one	X-2	Acetone-petroleum ether	0.585	406	695	249
	20β-Acetoxy-4-pregnen-3-one	X-3	Acetone-petroleum ether	0.438	307	702	251
	20β-Acetoxy-4-pregnen-3-one	ML-1		0.830	582	702	251
	20β-Acetoxy-4-pregnen-3-one	ML-3		0.468	332	701	251

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

units of adrenocorticotrophic 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 800 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 pregnanediol (56 μmoles) 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 CH_2Cl_2, and the solution was poured onto a 5-g column of alumina (containing 6% H_2O). The column was developed with 0.5% methanol in CH_2Cl_2, and the fractions containing pregnanediol 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 5β-pregnane-3,20-dione with CrO_3. Pregnanediol was dissolved in 0.5 ml of acetic acid, and a solution of CrO_3 (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 CrO_3 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 reaction mixture was slightly alkaline. The product was extracted into 150 ml of ether, and the ether solution was washed with 50 ml of 5% HCl, 50 ml of 5% NaOH, and finally with water. Evaporation left a residue which was chromatographed in thin layer chromatography System T-5. Pregnanedione (R_F 0.4) was extracted with chloroform and recrystallized from acetone-ligroin. Its specific activity is given in Table II. The total counts isolated as pregnanediol were 1300 cpm which corresponds to a conversion of 0.04%. (This is a minimum figure since no correction was made for endogenous pregnanediol.)

In order to compare this yield with that which might result from the administration of a natural precursor, pregnenolone (1×10^7 cpm, 0.5 nmole) was injected intravenously into a rabbit, and the pregnanediol excreted into the urine for the following 2 days was isolated. It contained 47,000 cpm which amounts to a yield of 0.5%.

Although Compound **9** was extensively metabolized to unidentified products by incubation with a viable rabbit liver homogenate, neither pregnenolone nor pregnanediol was formed from it under these conditions. Likewise, pregnenolone was not produced from the *t*-butyl analog **9** when the latter was refluxed in either acid (0.1 N HCl) or alkaline (0.5 N $NaHCO_3$) solution for ½ hour.

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 × *g* for 15 min, the supernatant was poured off and recentrifuged at 7000 × *g* for 20 min. The resulting mitochondrial pellet was suspended in 270 ml of the isolation medium and spun at 600 × *g* for 15 min; the supernatant was poured off and recentrifuged at 7000 × *g* for 20 min. The mitochondrial pellet was resuspended in the medium, and the suspension was centrifuged again for 20 min at 7000 × *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 periods with a 60-watt micro tip (Ultrasonics 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-^{3}H]5-pregnene-3β,20 diol, **9**, (5.36×10^6 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 $CaCl_2$, 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-^{3}H]5-pregnene-3β,20-diol, **9**, (2.27×10^6 cpm, 0.1 nmole) was added to three tubes (*a*, *b*, and *c*). 20α-[7α-^{3}H]Hydroxycholesterol (4.71×10^5 cpm, 1.8 nmoles) (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 reisolated carrier was characterized in the following manner.

Experiment IIa—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 liquors are given in Table II. The product of the second crystallization was acetylated 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 62,500 cpm recovered as pregnenolone or to a 1 2% yield.

Experiment IIb—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 cpm per μmole. After a second recrystallization from the same solvent, the value decreased to 17 cpm per μmole.

Experiment IIIa—After chromatography on Celite, System C-1, those fractions containing carrier pregnenolone (17.5 mg, 55 μmoles) 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 cpm per μmole) corresponds to a total recovery of 10,500 cpm as pregnenolone, a yield of 0.46%.

Experiment IIIb—Pregnenolone (16.3 mg, 52 μmoles, 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 μmoles) was then added, and heating was continued for 30 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 Na_2SO_4 and evaporated to dryness in a flash evaporator. The remaining residue was chromatographed on Celite with System C-1. Progesterone (11.3 mg, 36 μmoles, 242 cpm per μmole) 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 R_F of 0.5. The resulting sample of progesterone (specific activity 233 cpm per μmole) 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 μmoles) 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^{-3} M EDTA) containing 1 mg of 20β-hydroxy steroid dehydrogenase (Streptomyces hydrogenans-Sigma type II) and 100 mg (150 μmoles) 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 H_2O, dried over anhydrous Na_2SO_4, evaporated, and the residue was chromatographed in System T-1. The product, 20β-hydroxy-4-pregnen-3-one (8.6 mg, 27 μmoles), migrating with an R_F of 0.4 (progesterone R_F = 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 Na_2SO_4, and evaporated to dryness. The residue was chromatographed by thin layer chromatography in System T-3 (R_F = 0.4). The isolated 20β-acetoxy-4-pregnen-3-one (7.4 mg, 21 μmoles) 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 cpm per μmole), the yield of pregnenolone formed 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.).

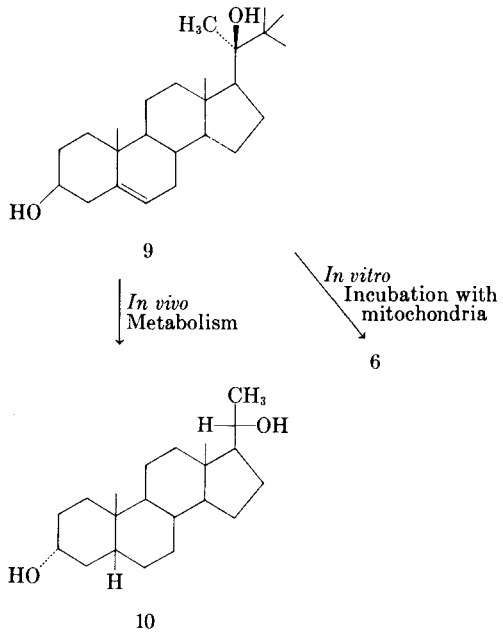
Experiment IIIc—After chromatography in System C-1, the fractions containing carrier pregnenolone (15.6 mg, 49 μmoles, added) were pooled and rechromatographed in System T-1. Pregnenolone, isolated after the incubation of the tritiated analog **9** with the heat-denatured enzyme, had an apparent specific activity of 4 cpm per μmole which corresponds to a conversion of less than 200 cpm (<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 μmoles, added), following incubation of 20α-[7α-^{3}H]hydroxycholesterol, was isolated by chromatography with System C-1 and was recrystallized from methanol to constant specific activity (2385 cpm per μmole). The number of counts per min recovered as pregnenolone was 120,500 cpm 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-pregnene-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 μmoles) had a specific activity of 311 cpm per mg. It was dissolved in 0.35 ml of acetic acid, the solution was heated to 53–55°, and 23 mg (230 μmoles) of crystalline CrO_3 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 Na_2SO_4, 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-



SCHEME I. Metabolism of the *t*-butyl analog **9** *in vivo* and *in vitro*.

due was chromatographed on tlc in System T-6, in which 7-ketopregnenolone acetate migrates with an R_F of 0.4. After elution of the steroid from the silica gel plate with methylene chloride-methanol (10:1) and evaporation of the solvents, the product was crystallized from aqueous methanol to yield needles (4 mg, 11 μmoles), m.p. 149–151°. It was identified as 7-ketopregnenolone acetate by comparison with an authentic sample; the corresponding infrared and ultraviolet spectra, melting point, and mobility on thin layer chromatography were all identical. 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 from which it was derived.

DISCUSSION

The analog, (20R)-20-*t*-butyl-5-pregnene-3β,20-diol, **9**, shown in this study to be a precursor of pregnenolone, was synthesized by addition of *t*-butyllithium to pregnenolone acetate in toluene at −70°. In the course of the condensation, partial hydrolysis of the acetate group occurred, resulting in the isolation of both Compound **9** and its 3β-acetate derivative. 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. 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 associated with the C-18 (0.90 ppm) and, especially, the C-21 (1.33) methyl groups of **9**, downfield relative to the corresponding data for cholesterol (0.66, 0.87, respectively) and its 20α (0.79, 1.17)- and 20β-hydroxy (0.78, 1.00) derivatives (20; see also Footnote 10 in Reference 21), suggest the α stereochemistry for the C-20 hydroxy group. Second, the infrared spectrum (KBr) of the acetate contains a *sharp* hydroxy band (3490 cm^{-1}) indicating that the C-20 tertiary hydroxy group is too hindered for intermolecular hydrogen binding. This pronounced feature is common to the infrared spectrum (KBr) of the 3β-acetate of 20α-hydroxycholesterol while, in the same region, the 20β isomer exhibits a characteristic broad band between 3600 and 3400 cm^{-1} [2]

[2] L. Bandy, O. Gonzales, and S. Lieberman, unpublished results.

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 20-hydroxy group in **9** has the R configuration while the 20-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 pregnanediol, **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 Δ^5-3β-hydroxy portion of the C_{19} steroidal nucleus (23), a common feature of **1**, **2**, and **9**.

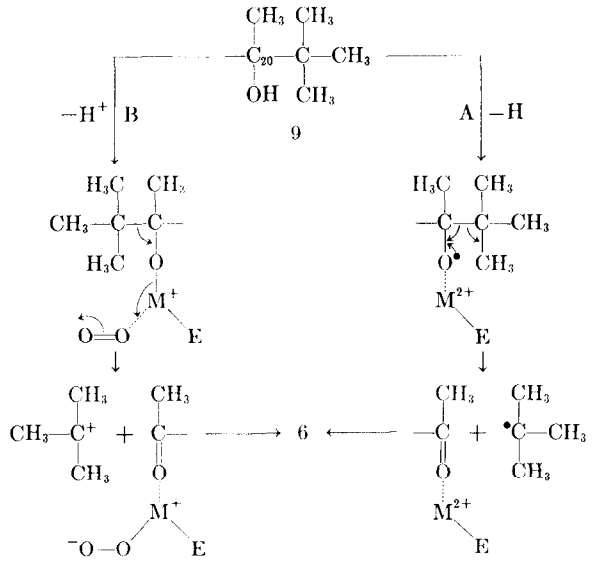
The poor yield of pregnenolone from **9** requires a close examination of the experimental conditions, particularly with regard to the *in vitro* incubations, to assure lack of interference by undesirable substances. It is apparent that contamination of the substrate with an isomer not fully substituted 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 commonly contain small amounts of the isobutyl isomer which could give rise to just such contamination. To avoid this possibility, a sample of *t*-butyl chloride that was free from primary and secondary isomers was prepared. Analytical grade *t*-butanol was oxidized exhaustively with permanganate to convert primary and secondary alcohols into their acids and ketones, respectively. 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° (literature b.p. 50.7°), was obtained. *t*-Butyllithium was prepared from this purified sample

of *t*-butyl chloride. These precautions would seem to preclude the possiblity 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, [7α-^{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 ^{3}H 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, [^{3}H]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 tritiated **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 20β-hydroxysteroid dehydrogenase to 20β-hydroxy-4-pregnen-3-one. The latter was characterized as its acetate. The pregnanediol isolated from the *in vivo* experiment was characterized as such and as its diketone, pregnanedione.

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 pregnanediol 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 pregnanediol, 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 irrelevant 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 pregnanediol (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 pregnanediol, is of little importance. On the other hand, the mechanism by which the conversion **9** → **6** occurs does deserve consideration, for its own sake and for what it may contribute to our understanding of the intimate details of the natural transformation of cholesterol into pregnenolone.

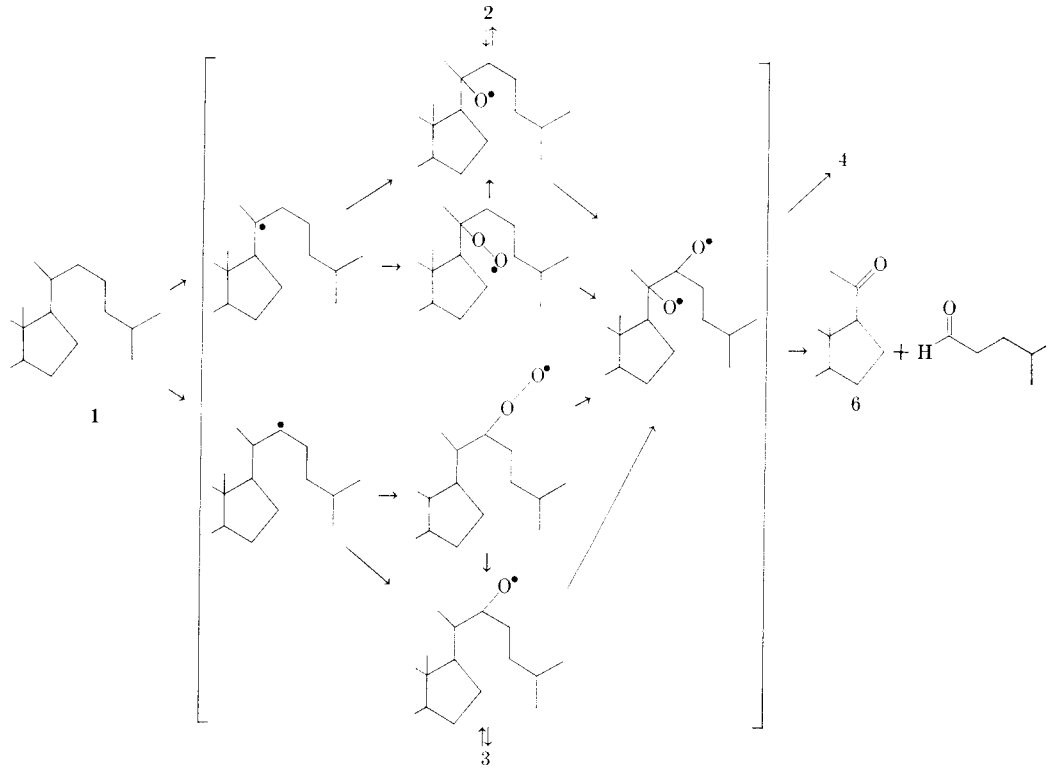


SCHEME II. Proposed radical (Path *A*) and ionic (Path *B*) mechanisms for the biosynthesis of pregnenolone (**6**) from the *t*-butyl analog **9**.

Possible Intermediates in Process: **9** → **6**—With the demonstration that the *t*-butyl analog **9** can serve as a precursor of pregnenolone, mechanisms[3] 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 (—CH_3 → —CH_2OH → —CHO → —COOH) and ionic decarboxylation. Facile loss of carbon dioxide is made possible by the presence of a β-carbonyl group or of a β,γ-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_2-labile, aliphatic carboxyl radical (RCO_2^- → $RCO_2\cdot$ → $R\cdot$ + 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.

[3] "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 C_4 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*-butyl group. In this way, free carbonium ion intermediates might not be formed.

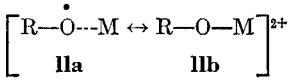
These greatly simplified radical and ionic schemes (Scheme II) each afford a rational mechanistic explanation for the oxidative cleavage of the *t*-butyl analog **9**. Therefore, it seems reasonable that side chain cleavage can occur without *prior* introduction of an oxygen function at C-22, although a mechanism in which carbon-carbon bond cleavage and C-22 oxygenation (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 (1) → 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. Oxygenation 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

[4] In the structure shown, a *solid line* between the metal and ligand atoms indicates a 2-electron bond (ionic, covalent, *etc.*) in which 1 electron each comes from the metal and ligand atoms; a *dashed line* indicates a 2-electron bond in which both shared electrons are donated by the ligand atom. The valence of the metal is determined by adding together the number of solid lines drawn to the metal atom and the number of charges on the complex; *e.g.* in the intermediate drawn in Path *B*, two solid lines and one positive charge indicate a total positive valence of three for the metal atom.

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 any single valence bond structure[4] (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.



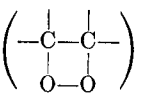
11a **11b**

Since the short lived intermediates proposed in Scheme III for the biosynthetic pathway from **1** to **6** are probably oxygenated steroid 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 alkoxy 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 C_8 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 C_6 hydrocarbon fragment. As previously stated, introduction of a second oxygen atom into a mono-oxygenated species may be concerted with cleavage of the C-20 to C-22 carbon-carbon bond, in which case the C_{21} and C_6 products, pregnenolone and isocaproaldehyde (29), would result without the intermediacy of a C_{27} 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 over-all pathway for this complex biosynthetic process remains speculative.

General nonradical mechanisms which are reasonable for mixed function oxidase systems, including the side chain cleavage enzymes, have been proposed and considered by Hamilton (28). Few chemical models exist for the position-specific and stereospecific biological oxidation of unactivated positions in saturated hydrocarbons (*e.g.* steroids). One system studied recently (30) is the oxidation of saturated hydrocarbons by ozone. This reaction is surprisingly stereospecific; yet the transition state possesses a high degree of radical character. Other model systems for mixed function oxidases seem to operate by transferring oxygen atoms via "oxenoid" mechanisms, analogous to the mechanisms of carbenoid 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 alkanes 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 (36) 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 Δ^5-unsaturated system in Ring *B* (37). In the cleavage of olefins to carbonyl compounds by singlet oxygen, dioxetanes



are presumed to be intermediates (38). In the decomposition of dioxetanes into carbonyl products, the intermediacy 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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