

Studies on the Biosynthetic Conversion of Cholesterol into Pregnenolone

SIDE CHAIN CLEAVAGE OF SOME 20-*p*-TOLYL ANALOGS OF CHOLESTEROL AND 20 α -HYDROXY-CHOLESTEROL*

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SUMMARY

Some 20-*p*-tolyl analogs of 20 α -hydroxycholesterol and cholesterol have been synthesized and tested as precursors of pregnenolone. As in the previously synthesized *t*-butyl analog, (20R)-20-*t*-butyl-5-pregnene-3 β ,20-diol, **C-22** in these synthetic compounds is completely substituted and therefore unavailable for biological hydroxylation (1). (20R)-20-(*p*-Tolyl)-5-pregnene-3 β ,20-diol, **1**, when incubated with an acetone powder preparation of bovine adrenal mitochondria forms pregnenolone and progesterone in good yield (more than 23%). (20S)-20-(*p*-Tolyl)-5-pregnen-3 β -ol, **2**, prepared by Raney nickel hydrogenation of **1**, when incubated in a similar fashion yields **1** in large quantity in addition to small amounts of pregnenolone and progesterone. This is the first reported instance of substantial *in vitro* 20 α -hydroxylation of a 22-desoxy substrate, natural or artificial. The styrene 20-(*p*-tolyl)-5,20-pregnadien-3 β -ol, **3**, made by dehydration of **1**, is not a substrate for side chain cleavage. The results support the previously advanced hypothesis that the true intermediates in the side chain cleavage of cholesterol are reactive complexes of a metalloenzyme and oxygenated steroidal species, and not free hydroxylated compounds. Two reaction mechanisms consistent with these findings are proposed.

in which the traditional side chain hydroxylated compounds, 20 α -hydroxycholesterol, 22R-hydroxycholesterol, and 20 α ,22R-dihydroxycholesterol, are not obligatory intermediates has been advanced (1). This new hypothesis considers the hydroxy compounds to be by-products resulting from as yet undefined reversible transformations of the true intermediates. Although their nature remains speculative, it seems reasonable to assume that the true intermediates might be short-lived, reactive complexes of oxygenated steroidal species and a metalloenzyme.

Support for this new hypothesis comes from experiments which demonstrated the metabolism, both *in vivo* in a rabbit and *in vitro* with bovine adrenal mitochondrial preparations, of (20R)-20-*t*-butyl-5-pregnene-3 β ,20-diol to pregnanediol and pregnenolone, respectively (1). This compound, a *t*-butyl analog of 20 α -hydroxycholesterol, is completely substituted at C-22 and thus cannot form a glycol analogous to 20 α ,22R-dihydroxycholesterol prior to cleavage of the C-20,22-bond. Oxygenation at C-22, if it occurs, is probably coincident with the breaking of the carbon-carbon bond.

To build still greater flexibility and assurance into experiments designed to explore further the nature of the side chain cleavage process, a series of analogs, **1**, of 20 α -hydroxycholesterol in which the isohexyl side chain had been replaced by a *p*-substituted phenyl ring were synthesized and tested as *in vitro* precursors of pregnenolone (2). Several features of these compounds seemed advantageous. (a) The effects of various electron-releasing and electron-withdrawing *p*-substituents (-R) on the rate of side chain cleavage could be compared. (b) Facile hydrogenation of the benzylic hydroxy group of **1** could yield **2** (Scheme 1), in which not only side chain cleavage but also 20 α -oxygenation might be studied. (c) The possible role of the styrene **3**, formed easily by dehydration of **1** (Scheme 1), in side chain cleavage could be investigated. (d) The aromatic fragment resulting from side chain cleavage would lend itself readily to identification and quantitation, thereby enabling the stoichiometry of the over-all reaction to be established. Some experiments with

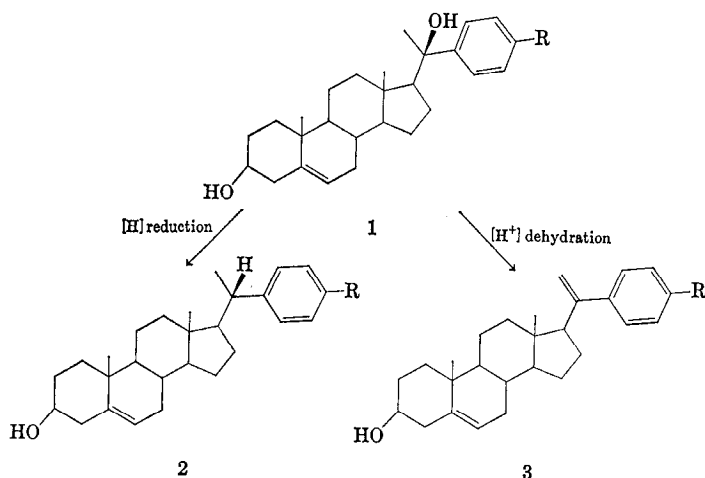
As an alternative to the previously proposed routes for the biosynthetic conversion of cholesterol¹ into pregnenolone, a scheme

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¹ Systematic names for steroids given trivial names in the text are: cholesterol, 5-cholesten-3 β -ol; 20 α -hydroxycholesterol, 5-cholestene-3 β ,20 α -diol; 22R-hydroxycholesterol, (22R)-5-cholestene-3 β ,22-diol; 20 α ,22R-dihydroxycholesterol, (22R)-5-cholestene-3 β ,20 α ,22-triol; pregnenolone, 3 β -hydroxy-5-pregnen-20-one;

pregnenolone acetate, 20-oxo-5-pregnen-3 β -yl acetate; progesterone, 4-pregnene-3,20-dione; pregnanediol, 5 β -pregnane-3 α ,20 α -diol; pregnenolone-3-acetate-20-methoxime, 20-methoxyimino-5-pregnen-3 β -yl acetate.

SCHEME 1
Structures of 20-aryl analogs



p-tolyl analogs 1, 2, and 3 ($R = \text{CH}_3$) are described in the present paper.

EXPERIMENTAL PROCEDURE

Melting points were determined on a Thomas-Hoover Melting Point Apparatus and are uncorrected. Infrared (ir) spectra were taken in micro potassium bromide discs with a Perkin-Elmer model 521 grating infrared spectrophotometer equipped with dual 4x reflecting beam condensers. Nuclear magnetic resonance (NMR) spectra were recorded with a Varian HA-100 spectrometer. Mass spectra were obtained with an MS-9 mass spectrometer and a computerized data system. Radioactive samples were counted in a Packard model 3375 liquid scintillation spectrometer (57% efficiency for ³H) to a counting error of less than $\pm 5\%$. Partition column chromatography was done as described by Kelly *et al.* (3). All solvents were distilled prior to use. Tracer [7α -³H]pregnenolone (New England Nuclear Corp., specific activity 25 Ci per mmole) was purified as previously described (1). Two types of silica gel were used for column chromatography; Silica Gel A (Woelm, 0.05 to 2.0 mm grade) was more active than Silica Gel B (Will Scientific). The latter type was used mainly for purification of the radioactive tracers.

Preparation of (20*R*)-20-(*p*-Tolyl)-5-pregnene-3 β ,20-diol, 1

Immediately after thoroughly oven-drying the glassware, a 2-liter three-necked round bottom flask, fitted with a reflux condenser, drying tube, 500-ml dropping funnel, gas inlet, magnetic stirrer, and heating mantle, was flushed with dry (Drierite) nitrogen gas for 30 min. Lithium ribbon (Matheson, Coleman & Bell) (17.5 g, 2.5 moles) was degreased with petroleum ether, dried with a paper towel, and cut into small pieces approximately 2 to 3 mm square with a pair of surgical scissors. The pieces were cut directly into the reaction flask under a continuous stream of nitrogen and then covered with 400 ml of freshly distilled (from lithium aluminum hydride) anhydrous ether. A solution of 123 ml (1 mole) of *p*-tolyl bromide (Eastman Organic Chemicals) in another 100 ml of anhydrous ether was put into the addition funnel, and a small portion was added to the flask. The reaction started spontaneously in 2 to 3 min. The remaining halide solution was added with continuous stirring at a rate necessary to maintain vigorous reflux over a period of about 2 hours. The mixture was heated for an additional 45 min under continuous reflux.

TABLE I

Chromatography systems

C, column-Celite partition system: 0.5 ml (normal) or 0.3 ml (reverse phase) of stationary phase per g of Celite; T, thin layer-Silica Gel GF; visualization: 3-keto- Δ^4 -steroids, ultraviolet light; 3β -OH- Δ^5 -steroids, Rhodamine G spray (0.02% in MeOH) followed by ultraviolet light (nondestructive) or phosphomolybdic acid (10% in EtOH) followed by warming to 100° (destructive). In C-1, each solvent was saturated with the other. Isooctane was the mobile phase, and methyl cellosolve was the stationary phase.

C-1	Isooctane-methyl cellosolve
C-2	Isooctane-methanol-water (10:9:1)
C-3	Toluene - isooctane - methanol - water - <i>n</i> - propyl alcohol (2:2:4:2:0.75) (reverse phase)
C-4	Toluene-isooctane-methanol- <i>t</i> -butyl alcohol-water (2:6:8:-1:3) (reverse phase)
C-5	Isooctane- <i>t</i> -butyl alcohol-methanol-water (10:2:7:1)
T-1	Benzene-ethyl acetate (1:1)
T-2	Benzene-ethyl acetate (2:1)
T-3	Benzene-ethyl acetate (3:1)
T-4	Benzene-isooctane-ethyl acetate (2:2:1)
T-5	Benzene-ethyl acetate (5:1)
T-6	Methylene chloride-acetone (95:5)
T-7	Cyclohexane-diethyl ether (3:2)
T-8	Isooctane- <i>t</i> -butyl alcohol (85:15)
T-9	Benzene-ethyl acetate (30:1)

A solution of 120 g (0.33 mole) of pregnenolone acetate (G. D. Searle) in 500 ml of dry benzene and 100 ml of dry ether was placed in the dropping funnel and slowly added to the *p*-tolyl-lithium solution over a period of 1 hour 45 min, while reflux was maintained throughout. During this period a large amount of an off-white precipitate formed gradually. After refluxing this mixture overnight, ice and water (about 1:1) were added to the flask to decompose the lithium compounds and excess lithium. The precipitate was collected by suction filtration, washed with ether, and dried. It was crystallized from pyridine-petroleum ether to yield about 100 g (74%) of small, colorless plates which gave a single spot ($R_F = 0.37$) after thin layer chromatography in System T-2 (Table I). The analytical sample, as well as those used in incubations, was recrystallized from aqueous ethanol. This compound was identified as (20*R*)-20-(*p*-tolyl)-5-pregnene-3 β ,20-diol by its infrared, NMR, and mass spectra and C-H analysis: m.p. 245–246° decomp.; infrared spectrum (micro KBr): 3560 (sharp, 20 α -OH), 3455 (broad, 3 β -OH), 1503 (aromatic C=C), 1046 (Δ^5 -3 β -OH), and 813 cm^{-1} (1,4-disubstituted aromatic ring); nuclear magnetic resonance (CDCl_3 , tetramethylsilane): δ 7.27 (d, 2, $J_{\text{ortho}} = 8$ Hz, aromatic H's meta to CH_3 group), 7.03 (d, 2, $J_{\text{ortho}} = 8$ Hz, aromatic H's ortho to CH_3 group), 5.25 (m, 1, H-6, vinyl), 4.28 (m, 1, 3-OH; disappeared after active proton exchange with CD_3OD), 3.85 (s, 1, 20-OH), 3.30 (m, 1, H-3 α , axial), 2.28 (s, 3, tolyl- CH_3), 1.56 (s, 3, H-21), 0.97 (s, 3, H-19), and 0.88 ppm (s, 3, H-18); mass spectrum (70eV): low resolution: 408 (0.1, parent), 390 (1.8, M- H_2O), 375 (0.2, M- H_2O - CH_3), 372 (0.1, M-2 H_2O), 357 (0.6, M-2 H_2O - CH_3), 274 (0.6, M- $\text{C}_9\text{H}_{11}\text{O}$ side chain), 256 (7.3, M- H_2O - $\text{C}_9\text{H}_{11}\text{O}$), 241 (1.4, M- H_2O - CH_3 - $\text{C}_9\text{H}_{11}\text{O}$), and 135 (100.0, base, p - CH_3 - C_6H_4 -C(CH_3)=OH⁺); high resolution: M^+ calculated, 408.3028; M^+ found, 408.3022.



Calculated: C 82.30, H 9.87
Found: C 82.18, H 9.75

Preparation of (20S)-20-(*p*-Tolyl)-5-pregnen-3 β -ol, 2

Raney nickel catalyst (W-2 type) was prepared according to the method of Mozingo (4) and stored under absolute ethanol.

A mixture of 170 mg (0.42 mmole) of **1** and 5 g of Raney nickel catalyst in 60 ml of absolute ethanol was made in a dried 100-ml round bottom flask fitted with a reflux condenser, drying tube, magnetic stirrer, and heating mantle. After refluxing for 3 hours, the reaction mixture was allowed to cool for a few minutes and then filtered through Celite while still warm. The filtrate was evaporated, leaving a white solid residue behind. This solid was chromatographed on a 20-g Silica Gel A column prepared in petroleum ether-benzene (80:20). The major product was eluted with benzene; the appropriate fractions were combined, evaporated, and the residue was crystallized from methanol-ether to give colorless plates, 140 mg (82%); m.p. 261–263° decomp.; R_f 0.49 (System T-2); infrared spectrum (micro KBr): 3450 (broad, 3 β -OH), 1508 (aromatic C=C), 1048 (Δ^5 -3 β -OH), and 812 cm⁻¹ (1,4-disubstituted aromatic ring); nuclear magnetic resonance (CDCl₃, tetramethylsilane): δ 7.04 (s, 4, aromatic H's), 5.33 (m, 1, H-6, vinyl), 3.53 (m, 1, H-3 α , axial), 2.56 (m, 1, H-20 α), 2.30 (s, 3, tolyl-CH₃), 1.62 (s, 1, 3 β -OH, disappeared after active proton exchange with CD₃OD), 1.23 (d, 3, H-21), 1.02 (s, 3, H-19), and 0.78 ppm (s, 3, H-18); mass spectrum (70 e.v.): low resolution: 392 (20.0, parent), 377 (0.8, M-CH₃), 374 (2.0, M-H₂O), 273 (6.2, M-C₉H₁₁ side chain), 255 (18.8, M-H₂O-C₉H₁₁), 119 (100.0, base, *p*-CH₃-C₆H₄-C⁺(H)-CH₃); high resolution: M⁺ calculated, 392.3079; M⁺ found, 392.3071.



Calculated: C 85.66, H 10.27

Found: C 85.78, H 10.17

Preparation of 20-(*p*-Tolyl)-5,20-pregnadien-3 β -ol, 3

A solution of 100 mg of *p*-toluenesulfonic acid in 100 ml of dry benzene was placed over 100 mg of anhydrous calcium chloride in a 250-ml Erlenmeyer flask. One hundred fifty milligrams of **1** were added to this solution and dissolved by warming to about 50°. After standing overnight, the solution was decanted, washed once with 200 ml of saturated bicarbonate solution, then three times with 100-ml portions of water, dried over Drierite, and evaporated to dryness. The solid residue was chromatographed on a 20-g Silica Gel A column prepared in petroleum ether. The product, 20-(*p*-tolyl)-5,20-pregnadien-3 β -ol, **3**, was eluted with 10% ether in benzene and crystallized from dilute methanol as colorless plates: m.p. 138–139°; infrared spectrum (micro KBr): 3280 (broad, 3 β -OH), 1612, 1555, and 1502 (conjugated aromatic C=C), 1050 (Δ^5 -3 β -OH), and 818 cm⁻¹ (1,4-disubstituted aromatic ring); nuclear magnetic resonance (CDCl₃, tetramethylsilane) δ 7.16 (d, 2, J_{ortho} = 8 Hz, aromatic H's meta to CH₃), 7.08 (d, 2, J_{ortho} = 8 Hz, aromatic H's ortho to CH₃), 5.33 (m, 1, H-6, vinyl), 5.14 (s, 1, H-21, vinyl methylene), 5.04 (s, 1, H-21, vinyl methylene), 3.48 (m, 1, H-3 α), 2.72 (t, 1, J = 9 Hz, H-17 α), 2.33 (s, 3, aromatic CH₃), 1.60 (s, 1, 3 β -OH, disappeared after active proton exchange with CD₃OD), 0.93 (s, 3, H-19), 0.55 ppm (s, 3, H-18); mass spectrum (70 e.v.): low resolution (empirical formula of fragments determined from high resolution mass spectrum) 390 (100.0, parent and base), 375 (5.6, M-CH₃), 372 (3.3, M-H₂O), 357 (20.3, M-CH₃-H₂O), 271 (3.8, M-C₉H₁₁ side chain), 253 (5.0, M-H₂O-C₉H₁₁ side chain), 229 (14.1, M-C₁₂H₁₇, loss of side chain and ring D), 171 (12.0, M-C₁₅H₂₃O), 159 (32.5, M-C₁₆H₂₃O), 158 (25.4, M-C₁₆-H₂O), 157 (10.2, M-C₁₆H₂₅O), 145 (56.2, M-C₁₇H₂₅O), 144 (48.8, M-C₁₇H₂₆O), 143 (24.0, M-C₁₇H₂₇O), 132 (14.5, M-C₁₈H₂₆O),

131 (11.1, M-C₁₈H₂₇O), 129 (29.5, M-C₁₈H₂₉O), 119 (33.7, *p*-CH₃-C₆H₄-C⁺=CH₂); high resolution: M⁺ calculated, 390.2920; M⁺ found, 390.2900.

Preparation of (20R)-20-(*p*-Tolyl)-5-[7 α -³H]pregnene-3 β ,20-diol, 1

p-Tolyl bromide (Eastman) was distilled, and the fraction boiling at 182.7–182.8° (literature: b.p. 184–185°) was used for the preparation of *p*-tolyllithium.

A dry 25-ml two-necked round bottom flask, equipped with a reflux condenser, drying tube, and gas inlet, was flushed completely with dry nitrogen gas, and about 1 g of lithium ribbon (cleaned and cut up as described before) was put into the flask under about 15 ml of freshly distilled (from lithium aluminum hydride) anhydrous ether. After adding approximately 0.5 ml of distilled *p*-tolyl bromide and mixing the reagents, the reaction began within 5 min. Reflux was continued for another 40 min. Then about 10 ml of this solution of *p*-tolyllithium were added to a solution of 1.0 \times 10⁹ cpm of [7 α -³H]pregnenolone in 5 ml of dry benzene in a separate 25-ml round bottom flask. This mixture was refluxed overnight. After cooling the reaction mixture, about 10 ml of water were added to decompose the excess lithium reagent. Following removal of the aqueous solution, the organic layer was washed three times with 10-ml portions of water, diluted to a volume of about 200 ml with benzene-methanol (9:1), and stored in the dark at 4° until the start of the purification procedure.

The solvent was evaporated under reduced pressure, and the residue was chromatographed on a 20-g Silica Gel B column prepared in benzene. After passing 500 ml of benzene and 500 ml of benzene-ethyl acetate (99:1) through the column, the desired product (8.0 \times 10⁸ cpm) was eluted with benzene-ethyl acetate (97.5:2.5). This material was then rechromatographed on Celite in System C-1 (Table I) where it was eluted as a symmetrical peak (6.0 \times 10⁸ cpm in holdback volumes (HBV) 6 to 8. Upon acetylation of this tracer overnight at room temperature in pyridine-acetic anhydride (2:1), and, after removal of the excess reagents by evaporation, the residue was chromatographed in the reverse phase System C-3. The radioactivity eluted in HBV 8 to 14 (6.0 \times 10⁸ cpm) was chromatographed again on a 15-g column of alumina (containing 3% H₂O) prepared in petroleum ether. By using increasing amounts of benzene in petroleum ether, the radioactive acetate (4.8 \times 10⁸ cpm) was finally eluted in benzene-petroleum ether (4:1). Saponification of this acetate was then done overnight at room temperature in 90% methanol-10% aqueous sodium carbonate solution, and the product was purified by Celite chromatography in System C-2, whereupon 4.7 \times 10⁸ cpm eluted as a symmetrical peak in HBV 4 to 6. To confirm its radiochemical purity, a portion of this material was combined with unlabeled **1** and crystallized from acetone-petroleum ether. The data in Table II show that the initial specific activity of the mixture was maintained through two crystallizations. This criterion was taken as proof of radiochemical homogeneity.

Preparation of (20S)-20-(*p*-Tolyl)-5-[7 α -³H]pregnen-3 β -ol, 2

A solution of 1.0 \times 10⁸ cpm of (20R)-20-(*p*-tolyl)-5-[7 α -³H]-pregnene-3 β ,20-diol, **1**, in 15 ml of absolute ethanol was put into a 25-ml round bottom flask. Two hundred milligrams of W-2 Raney nickel catalyst in 2 ml of absolute ethanol were added, and the mixture was refluxed for 3 hours and allowed to cool overnight. After filtering the reaction mixture through Celite, the solvent was evaporated, and the residue was applied to a 20-g Silica Gel B column in heptane-benzene (4:1). The major

TABLE II
Crystallization data

Sample	Fraction ^a	Solvent ^b	Amount		Specific Activity	
			mg	cpm	cpm/mg	cpm/ μ mole
Purified Tracers:						
(20R)-20-(p-tolyl)-5-pregnene-3 β ,20-diol, <u>1</u>	X-0		0.344	1940	5640	2300
	X-1	A-P	0.560	3120	5570	2270
	X-2	A-P	0.807	4580	5680	2320
	ML-2		0.661	3660	5540	2260
(20S)-20-(p-tolyl)-5-pregnen-3 β -ol, <u>2</u>	X-0		0.910	2280	2500	982
	X-1	A-P	0.864	2140	2480	973
	ML-1		0.820	2060	2510	985
	X-2	A-P	0.786	1960	2490	976
	ML-2		0.503	1260	2510	984
20-(p-tolyl)-5,20-pregnadien-3 β -ol, <u>3</u>	X-0		0.484	1390	2880	1130
	X-1	A-P	0.704	1930	2750	1070
	ML-1		0.300	540	1810	710
	X-2	A-P	0.383	1110	2900	1130
	X-3	A-P	0.489	1390	2850	1110
Incubation I:						
Pregnenolone	X-1	A-P	0.743	7990	10750	3400
	ML-1		0.809	9050	11190	3540
	X-2	A-P	0.728	7640	10500	3320
	ML-2		0.867	9620	11100	3510
	X-3	A-P	0.868	8970	10340	3270
	ML-3		0.680	7310	10750	3400
	X-4	A-P	0.944	9990	10580	3340
	ML-4		0.676	7360	10890	3340
Pregnenolone-3-acetate	X-1	M	0.952	8810	9250	3310
	ML-1		0.806	7350	9120	3270
	X-2	M	0.518	4630	8940	3200
	ML-2		0.844	7780	9220	3300
20 β -hydroxy-4-pregnen-3-one	X-0		0.822	5780	7030	2220
	X-1	A-P	0.936	6630	7080	2240
	ML-1		0.935	6420	6870	2170
	X-2	A-P	0.865	6160	7130	2250
	ML-2		0.961	6840	7120	2250
20 β -acetoxy-4-pregnen-3-one	X-1	M	0.542	3550	6550	2340
	ML-1		0.468	2620	5600	2000
	X-2	M	0.482	3120	6470	2320
	ML-2		0.279	1810	6490	2320
Incubation II:						
(20R)-20-(p-tolyl)-5-pregnene-3 β ,20-diol, <u>1</u>	X-1	A-P	0.691	586	848	346
	ML-1		0.984	1100	1120	456
	X-2	A-P	0.805	656	815	333
	ML-2		0.675	587	870	355
	X-3	A-P	0.873	708	811	331
	ML-3		0.456	386	846	345
	X-4	A-P	0.647	517	799	326
	ML-4		0.141	111	787	321
20-(p-tolyl)-5,20-pregnadien-3 β -ol, <u>3</u>	X-1	A-P	0.640	530	828	323
	ML-1		0.405	325	802	313
Pregnenolone-3-acetate	X-0		0.905	889	761	272
	X-1	M	0.835	628	752	269
	ML-1		0.912	744	816	292
	X-2	M	0.919	711	774	277
	ML-2		0.892	684	767	275
Pregnenolone-3-acetate-20-methoxime	X-1	M	0.696	496	712	276
	ML-1		0.652	404	619	240
	X-2	M	0.760	550	723	280
	ML-2		0.567	394	694	269
Progesterone	X-1	A-P	0.906	174	163	51
	ML-1		0.445	81	124	39
	X-2	A-P	0.918	172	159	50
	X-3	A-P	0.903	170	160	50
20 β -hydroxy-4-pregnen-3-one	X-1	A-P	0.985	149	152	48
	ML-1		0.097	13	131	41
20 β -acetoxy-4-pregnen-3-one	X-1	A-P	0.944	130	138	49
	ML-1		0.189	21	111	40
20 β -acetoxy-4-pregnene-3-methoxime	X-1	M	0.672	83	124	48

^a X-0 = initial mixture before crystallization; X-n = crystalline product from the nth crystallization; ML-n = residue left in mother liquor from the nth crystallization.

^b A = acetone; P = petroleum ether (30-60°); M = methanol.

product (5.9×10^7 cpm) which eluted in heptane-benzene (1:1) was then chromatographed in System T-6 on a silica gel plate impregnated with silver nitrate. That portion of the plate which corresponded to the area where a standard sample of **2** had migrated was extracted with chloroform, and the extract was chromatographed on Celite in the reverse phase System C-4. A

symmetrical peak of radioactivity appeared in HBV 3 to 4, and it was chromatographed again on a 15-g Silica Gel B column prepared in heptane. The product (2.7×10^7 cpm) was eluted in benzene-heptane (7:3). Its radiochemical purity was demonstrated by crystallization with unlabeled carrier **2** from acetone-petroleum ether (Table II).

Preparation of 20-(*p*-Tolyl)-5,20-[7 α -³H]pregnadien-3 β -ol, 3

A mixture of 1×10^8 cpm of (20R)-20-(*p*-tolyl)-5-[7 α -³H]-pregnene-3 β ,20-diol, 20 mg of *p*-toluenesulfonic acid, and 20 mg of anhydrous calcium chloride in 20 ml of dry benzene was warmed to about 50° and allowed to stand overnight. After decanting the solution and washing it once with 25 ml of saturated sodium bicarbonate solution and three times with 20-ml portions of water, it was diluted to a volume of 200 ml with benzene-methanol (9:1). Evaporation of the solvents under reduced pressure (which also served to dry the sample by azeotropic distillation of any water present) left a residue which was chromatographed on a 20-g Silica Gel B column starting with heptane-benzene (4:1). The product (6.3×10^7 cpm) appearing in fractions of heptane-benzene (2:3) was shown to be radiochemically pure by diluting a portion of it with unlabeled 3 and crystallizing the mixture from aqueous methanol (Table II).

Preparation of Bovine Adrenal Mitochondrial Acetone Powder

Fresh, defatted, demedullated, bovine adrenocortical tissue was homogenized at 4° (pH 7.4) in a medium (20% (weight of tissue per volume of medium)) which was 0.25 M in sucrose, 0.001 M in tris(hydroxymethyl)aminomethane hydrochloride (Tris), and 0.001 M in EDTA, and thereafter the pH was adjusted back to 7.4 with 1 N KOH. After centrifuging this homogenate at $650 \times g$ for 10 min, the supernatant was decanted, and the pellet was resuspended in the medium (10% of the original volume) and spun at $650 \times g$ for 10 min. The resulting supernatant was combined with the first supernatant, and the mixture was centrifuged at $8000 \times g$ for 15 min. This new pellet was washed twice by resuspension in 200 ml of the isolation medium and centrifugation at $8000 \times g$ for 15 min. Then the pellet was resuspended in the medium and spun for 15 min at $650 \times g$. The supernatant so obtained was centrifuged at $10,000 \times g$ for 15 min, and the resulting mitochondrial pellet was suspended in water; the suspension was frozen and lyophilized (5). After taking up the solid residue in cold (−20°) acetone, the mixture was filtered, and the powder was washed with ether at −20°, dried under vacuum, and stored, still at −20°.

Incubation Experiments

General Procedure—For an incubation experiment, 200 mg of the acetone powder was suspended in 10 ml of 0.1 M Tris buffer (pH 7.4) and sonicated at 60 watts with a micro tip (Ultrasonics Inc., model W 1850) four times for 30-s periods at 30-s intervals. An ice bath was used to maintain the temperature at 0–4°. After sonification, the mixture was centrifuged at $10,000 \times g$ for 15 min. The supernatant was decanted, and a portion of it was introduced into each incubation tube as described below. For the control experiments, the enzyme was inactivated by boiling some of this supernatant for 15 to 20 min and then cooling it to room temperature with an ice bath just before use.

A typical incubation was done in the following way. The appropriate substrate was added in equal amounts in a low boiling solvent such as acetone to each of two tubes containing 0.1 ml of propylene glycol. After mixing the solvents, the lower boiling solvent was removed by evaporation. To these solutions was added 0.8 to 1.0 ml of 0.1 M Tris buffer (pH 7.4) with thorough mixing. Next, each tube received 0.5 ml of 0.1 M phosphate buffer (pH 7.4) containing a NADPH-generating system (12.5 mg (41.2 μ moles) of glucose 6-phosphate, 3.75 mg (5 μ moles) of NADP, 2.5 units of glucose 6-phosphate dehydrogenase (Sigma), and 0.05 ml of 0.1 M $MgCl_2$). Finally, 2 ml of the

supernatant described above (on the average, at least 10 mg of protein, as determined by the method of Groves *et al.* (6), and 3 nmoles of cytochrome P-450 as determined by the method of Omura and Sato (7)) were added to one tube, while the control tube received 2 ml of the supernatant which had been boiled. The total volume of liquid in each tube was adjusted to 5 ml with more 0.1 M Tris buffer solution, and the mixtures were shaken in air at 37° for 30 min. After stopping the incubations with the addition of 20 ml of methanol, methanolic solutions of appropriate carrier steroids were added. The mixtures were filtered to remove the precipitated protein, diluted to 300 ml with benzene, and partitioned with 50 ml of water. The water layer was separated and washed with another 50 ml of benzene. The benzene fractions were combined, evaporated, and the residue was chromatographed.

Incubation I—(20R)-20-(*p*-Tolyl)-5-[7 α -³H]pregnene-3 β ,20-diol, 1 (2.75×10^6 cpm, 87.7 pmoles), was incubated according to the procedure described above; pregnenolone (3.94 mg, 12.5 μ moles) and progesterone (3.62 mg, 11.5 μ moles) were added as carriers. The organic extract was chromatographed on Celite in System C-2 whereby the pregnenolone (holdback volumes (HBV) 7 to 9) and progesterone (HBV 3 to 4) carriers were separated. After pooling the appropriate fractions and evaporating the solvent, the pregnenolone residue was chromatographed in System C-1, and the pregnenolone resisolated from HBV 8 to 10 was acetylated (in pyridine-acetic anhydride (2:1), 50°, 1.5 hours) and purified by reverse phase chromatography in System C-3. This pregnenolone acetate sample was twice chromatographed on silica gel plates, first using System T-5 and then System T-7, and saponified (in methanol-aqueous saturated Na_2CO_3 (90:10), 50°, overnight). The resulting pregnenolone was purified by thin layer chromatography (System T-1); the 2.56 mg (67%) recovered was mixed with 20.6 mg of unlabeled pregnenolone (total 73.3 μ moles) and crystallized to constant specific activity (Table II). Again this pregnenolone was acetylated and purified by thin layer chromatography (as above) and crystallized twice; the specific activities of the crystals and residues left in the mother liquors are shown in Table II. The specific activity of the crystallized pregnenolone corresponds to a yield of 3.22×10^5 cpm (10.3 pmoles) or 11.7%.

The progesterone residue was chromatographed once in System C-1 (HBV 2 to 3), once in System T-2, then in System T-8, and finally was reduced enzymically with 20 β -hydroxysteroid dehydrogenase using the procedure previously described (1). Following thin layer chromatography of the product, 20 β -hydroxy-4-pregnen-3-one, in System T-1 (1.66 mg recovered) and the addition of 19.3 mg of cold carrier, the material (66.3 μ moles total) was crystallized twice (Table II). Acetylation of this sample gave 20 β -acetoxy-4-pregnen-3-one which was purified by thin layer chromatography in System T-4; the specific activity of this acetate remained constant through two crystallizations (Table II). The yield of progesterone was 3.24×10^5 cpm (10.3 pmoles) or 11.8%.

Thus, in this incubation the total yield of these two steroidal products from the side chain cleavage of 1 was 23.5%.

Incubation II—Following termination of the incubation of (20S)-20-(*p*-tolyl)-5-[7 α -³H]pregnen-3 β -ol, 2 (2.25×10^6 cpm, 71.8 pmoles), 1 (4.2 mg, 10.3 μ moles), pregnenolone (4.61 mg, 14.6 μ moles), and progesterone (4.1 mg, 13.1 μ moles) were added as carriers, and the extract was chromatographed on Celite in System C-1. In this system, 1 is eluted in HBV 11 to 16, pregnenolone in HBV 8 to 10, and progesterone in HBV 2 to 3.

The resisolated carrier 1 was acetylated in the usual way, puri-

fied by chromatography in the reverse phase System C-3 (HBV 10 to 13), then once each in thin layer chromatography Systems T-5 and T-7, and finally eluted from an alumina (3% H₂O) column with benzene. After one more purification by thin layer chromatography in System T-5, the sample (15% recovered) was mixed with 25 mg (55.6 μ moles) of pure 3 β -acetoxy-(20R)-20-(*p*-tolyl)-5-pregnen-20-ol. This acetate was then saponified, chromatographed once in System T-2, and crystallized to constant specific activity (Table II). Finally, this sample of **1** was dehydrated to form **3** (in benzene with a trace of *p*-toluenesulfonic acid, room temperature, overnight) and crystallized once (Table II). Based on the specific activity of the purified product, the total yield of **1** was 1.39×10^5 cpm (4.4 pmoles) or 6.2%. Yields of **1** from other incubations of **2** ranged as high as 21%.

The pregnenolone sample was acetylated and chromatographed three times, first in reverse phase System C-3 and then in Systems T-5 and T-7. This purified pregnenolone acetate (3.50 mg, 67% recovered) was mixed with 19.6 mg of carrier (total 73.1 μ moles) and crystallized twice (Table II). Then the sample was converted into pregnenolone-3-acetate-20-methoxime (in pyridine with methoxyamine hydrochloride, 60°, 5 hours) which was purified in System T-9. Its specific activity remained constant through two crystallizations (Table II). A total of 2.57×10^4 cpm (0.82 pmoles) or a 1.1% yield of pregnenolone was obtained.

The progesterone carrier, after chromatography in System C-1 and two thin layer chromatography systems, did not contain a significant amount of radioactivity. However, in other incubations of **2** a small yield of radioactive progesterone was recovered. For example, after an incubation of 2.0×10^6 cpm (63.8 pmoles) of **2**, 3.98 mg (12.7 μ moles) of progesterone were added as a carrier, the incubation extract was chromatographed in System C-1, and the reisolated progesterone was purified in three stages, first by Celite chromatography in System C-2, second by thin layer chromatography in System T-2, and finally by thin layer chromatography in System T-8. Then an additional 20 mg of unlabeled progesterone were admixed with the 3.40 mg (85%) recovered, and the sample (total 74.5 μ moles) was crystallized three times (Table II). The progesterone was then reduced with 20 β -hydroxysteroid dehydrogenase to 20 β -hydroxy-4-pregnen-3-one which in turn was acetylated to form 20 β -acetoxy-4-pregnen-3-one, and finally this acetate was converted into the 20 β -acetoxy-4-pregnene-3-methoxime derivative. Each of these three derivatives was purified by thin layer chromatography and crystallized once. Their specific activities as shown in Table II remained constant. The yield of progesterone from this incubation was 4400 cpm (0.14 pmole) or 0.22%.

Incubation III—Pregnenolone (6.78 mg, 21.5 μ moles), progesterone (6.31 mg, 20.1 μ moles), and **1** (5.27 mg, 12.9 μ moles) were added as carriers following the incubation of 9.95×10^6 cpm (317 pmoles) of 20-(*p*-tolyl)-5,20-[7 α -³H]pregnadien-3 β -ol, **3**. Chromatography of the extract in System C-1 separated the three carrier fractions, and each was worked up in the following way.

The pregnenolone was eluted from System C-2, acetylated, and, after chromatography of this acetate in the reverse phase System C-3 and thin layer chromatography System T-4, the 5.6 mg (15.6 μ moles, 72.9%) of carrier recovered contained a total of only 14 cpm of radioactivity. This amount was considered insignificant, and the sample was not purified any further.

After reisolation, the progesterone sample was subjected to a treatment which would alter materially any compound, similar in polarity to progesterone, containing a labile hydroxy group without affecting progesterone itself (in benzene with a trace of *p*-toluenesulfonic acid, room temperature, overnight) and chro-

matographed first in System C-2 and then in System T-2. Following a second treatment with *p*-toluenesulfonic acid under the same conditions as above, the sample was eluted from System C-5 in HBV 2 to 3 and finally run in System T-8. Only 53 cpm remained in the 4.38 mg (13.9 μ moles, 69.4%) of the progesterone carrier recovered at this stage. No further effort was made to determine whether or not this radioactivity was actually associated with the carrier.

The sample containing carrier **1** was chromatographed in System C-2, then acetylated and purified in the reverse phase System C-3 and thin layer chromatography System T-4. At this stage, the acetylated carrier (3.93 mg, 8.7 μ moles, 67.5% recovered) contained 154 cpm. This amount was deemed insignificant, and no further purification was attempted.

Control Incubations—In each of the three incubation experiments just described, a control incubation was run using identical conditions except that the mitochondrial preparation had been boiled before use. Each control incubation was worked up in the same way as its counterpart. In every case, no significant amount of radioactivity remained with the appropriate carriers after their purification.

DISCUSSION

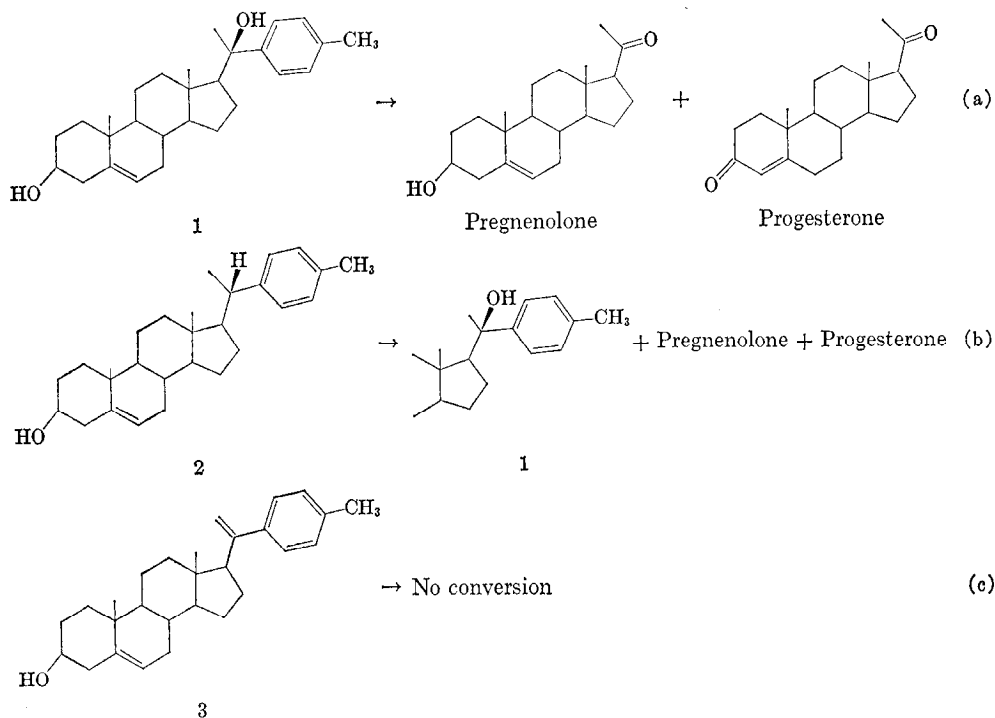
The *p*-tolyl analog of 20 α -hydroxycholesterol, (20R)-20-(*p*-tolyl)-5-pregnene-3 β ,20-diol, **1**, is prepared simply by the addition of *p*-tolyllithium to pregnenolone acetate, followed by hydrolysis and saponification of the 3-acetate to the corresponding hydroxy group. Stereospecific hydrogenation of **1** with W-2 Raney nickel gives the *p*-tolyl analog of cholesterol, (20S)-20-(*p*-tolyl)-5-pregnen-3 β -ol, **2**. Dehydration of **1** under extremely mild, acidic conditions yields exclusively the $\Delta^{20,21}$ -styrene, **3**. None of the $\Delta^{17,20}$ -olefin is formed. The structures and stereochemistry of **1**, **2**, and **3** were confirmed by thorough spectral (ir, NMR, and mass) analysis.

Radioactive tracers **1**, **2**, and **3** were prepared from [7 α -³H]-pregnenolone according to the same synthetic schemes as their unlabeled counterparts. They were rigorously purified by a variety of chromatographic procedures so as to exclude any possible contaminants, especially pregnenolone, which might have invalidated the results of the incubations. Radiochemical homogeneity of these tracers was demonstrated by the retention of the initial specific activity through several crystallizations of a mixture of an aliquot of the tracer with its corresponding unlabeled compound; the data are shown in Table II.

Incubation of analog **1** with an acetone powder preparation of bovine adrenal mitochondria produces both pregnenolone and progesterone in good yield (Scheme 2a). When the hydrogenated analog **2** is incubated with the same mitochondrial preparation under the same conditions, a large quantity of the hydroxylated product **1** is formed in addition to smaller amounts of pregnenolone or progesterone, or both (Scheme 2b). On the other hand, the styrene **3** is not converted by this enzyme preparation into any C₂₁-steroid ketones nor is it hydroxylated at C-20 (Scheme 2c). Control incubations of **1**, **2**, and **3** with boiled mitochondria did not show any conversion of the substrates into the products isolated from the ordinary incubations, thus demonstrating both that the observed metabolic transformations were indeed enzymically catalyzed and that the tritiated aryl derivatives were not contaminated with radioactive pregnenolone.

Each of the radioactive products from the incubation experiments performed was exhaustively chromatographed, derivatized several times, and rechromatographed after every chemical

SCHEME 2
In vitro incubations of *p*-tolyl analogs with acetone powder of bovine adrenal mitochondria



modification, and each product or derivative had its identity proven by reverse isotope dilution analysis, *i.e.* by mixing the isolated tracer with the appropriate unlabeled carrier and crystallizing this mixture to constant specific activity. Pregnenolone was characterized by acetylation, saponification, reacetylation, and formation of its 20-methoxime-3-acetate derivative. Progesterone was converted enzymically into 20 β -hydroxy-4-pregnen-3-one; this compound was in turn acetylated and transformed into 20 β -acetoxy-4-pregnene-3-methoxime. The enzymically formed analog 1 was acetylated, saponified, and dehydrated to form 3. Constant specific activities were maintained throughout all the chromatographic and derivatization procedures, as shown by the data in Table II. Thus, the identities of the products of each incubation have been proven.

Progesterone, formed in both Incubation I and Incubation II, probably arose through the action of some Δ^5 -3 β -hydroxysteroid isomerase-dehydrogenase on the pregnenolone initially produced via the side chain cleavage process. The amount of the isomerase-dehydrogenase present varied from preparation to preparation, as evidenced by the varying yields of progesterone in incubations using different batches of mitochondria.

Since 1 is so readily dehydrated under very mildly acidic conditions to give 3, it was necessary to eliminate the possibility that the olefin 3 was first being formed from 1 and then acting as the true substrate for the side chain cleavage enzyme system. The results of Incubation III conclusively demonstrate that this was not the situation. Not only is 3 itself not a precursor *in vitro* of pregnenolone or progesterone; it also is incapable of being hydroxylated at C-20 to form 1. This experiment would also seem to rule out other types of compounds which could conceivably form from 3, such as a C-20, 21-epoxide, from consideration as possible precursors of pregnenolone.

The formation of the 20 α -hydroxy-20-*p*-tolyl analog 1 in high yield from the *in vitro* incubation of 2 is significant for two reasons. This is the first reported unequivocal example of the *in*

vitro 20-hydroxylation of any 22-desoxy substrate, natural or artificial. But more importantly, in light of the demonstration that the aryl side chain can be cleaved to form the 20-ketone and that the amount of 1 formed from 2 (representing 20 α -hydroxylation) is much greater than the combined amount of pregnenolone and progesterone (representing side chain cleavage) isolated from the same incubation, it appears that in this case the C-20 position is the first to be oxygenated.

This observation is in contrast to the current thought on the mechanism of the side chain cleavage of cholesterol in which it is considered that C-22 is oxygenated prior to C-20 on the biosynthetic route to pregnenolone (8). This view has been based largely upon the assumption that side chain hydroxylated compounds are true intermediates in the cleavage process and upon three experimental observations: first, that 22R-hydroxycholesterol is formed from radioactive cholesterol in an *in vitro* incubation faster than is 20 α -hydroxycholesterol; second, that radioactive 22R-hydroxycholesterol is converted *in vitro* into pregnenolone at a faster rate than is 20 α -hydroxycholesterol; and third, that 22R-hydroxycholesterol is isolated in far greater abundance from adrenal extracts than is 20 α -hydroxycholesterol.

However, these facts can just as easily support the opposite conclusion if it is assumed that 22R-hydroxycholesterol and all other side chain hydroxylated cholesterol derivatives are not genuine intermediates on the path to pregnenolone. Rather these free hydroxy compounds may be by-products of some reversible biosynthetic transformations (*e.g.* hydrolysis) of the true intermediates, or, perhaps, they may even arise chemically from these intermediate complexes as artifacts of the procedure used for their isolation. Then, the observation that 22R-hydroxycholesterol is an excellent precursor of pregnenolone *in vitro* may be considered as merely reflecting the necessity of 22-oxygenation for side chain cleavage of cholesterol and the preference of the enzyme for performing the initial side chain oxygenation at C-20.

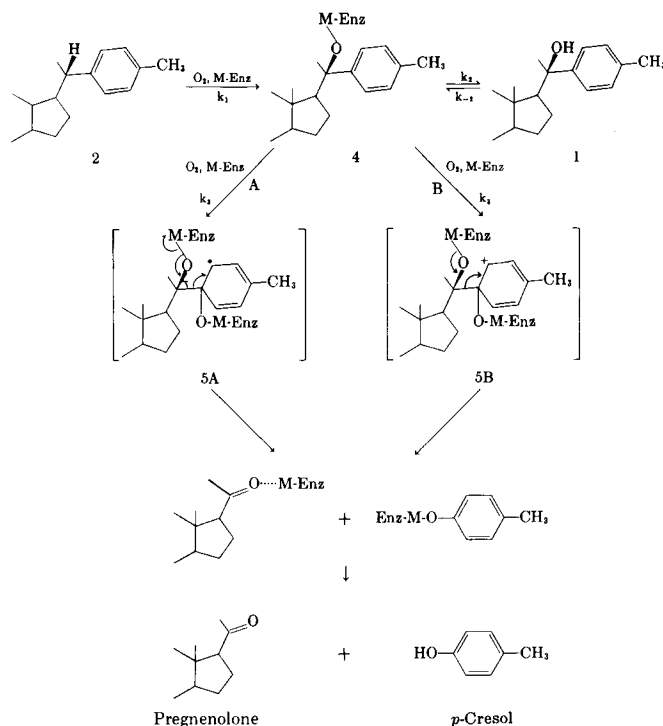
In addition, the failure to isolate no more than trace amounts of 20 α -hydroxycholesterol from adrenal extracts and its slower rate of conversion *in vitro* into pregnenolone when compared to that of 22R-hydroxycholesterol may indicate (a) that the true intermediate is some type of 20-oxygenated steroid-enzyme complex, formed directly from cholesterol, which is rapidly converted into pregnenolone; (b) that the interconversion of this intermediate and 20 α -hydroxycholesterol may occur at a correspondingly much slower rate; and (c) that 22-oxygenation may be secondary to, and, in addition, may be kinetically slower than, 20-oxygenation in the sequence of events leading to cholesterol side chain cleavage. This conversion of artificially introduced 20 α -hydroxycholesterol into an intermediate complex may still be faster than the *in vitro* formation of the same or a similar intermediate from cholesterol, thus explaining why 20 α -hydroxycholesterol is a better precursor *in vitro* of pregnenolone than is cholesterol. All this reasoning is consonant with the mechanistic situation proposed below for the *in vitro* metabolism of **1** and **2**.

Assuming that the same enzyme cleaves the side chain of both cholesterol and the synthetic aryl and *t*-butyl analogs, then the mechanisms for each of these processes must be similar. Of course, in the case of the artificial analog **2**, oxygenation first at C-20 is favored to a somewhat greater extent when compared to cholesterol because the chemical reactivity of C-20 is slightly enhanced by its benzylic character and because oxygenation at C-22 requires interruption of the aromatic system. But, again from a chemical standpoint, it is certainly logical to assume that oxygenation of the tertiary C-20 position in cholesterol would be faster than oxygenation of the secondary C-22 position. The very fact that side chain cleavage of the unnatural analogs occurs indicates a certain lack of specificity of the enzyme involved for the structure of the steroid side chain, but not necessarily for the order in which oxygen is introduced into the side chain. However, the isolation of 22R-hydroxycholesterol from adrenal extracts and incubations suggests that the side chain cleavage enzyme may not be entirely specific with respect to the order in which the side chain is oxygenated. Thus, that a single sequence for oxygenation of the C-20 and C-22 positions in cholesterol prior to scission of the C-20,22-bond exists has not been established with certainty, but the present experiments uphold the contention that C-20 may be the initial site of oxidative attack.

Oxygenation and side chain cleavage of cholesterol are catalyzed by a cytochrome P-450 enzyme system having the characteristics typical of a mixed function oxidase. Mixed function oxidases essentially catalyze the transfer of a single atom of oxygen from an oxygen molecule to a substrate by what Hamilton (9, 10) has termed an oxenoid-type reaction. The active oxygen species in these reactions may be either electrophilic or radical in nature. If it can be assumed that the cholesterol side chain cleavage enzyme is responsible for the side chain cleavage of the *p*-tolyl analogs observed in the present experiments, then two reasonable mechanisms for these transformations may be envisioned (Scheme 3). Common to both mechanisms is the intermediacy of transient, reactive complexes of the oxygenated steroidal species and the metalloenzyme (M-Enz). In neither mechanism are free hydroxylated compounds considered to be true intermediates.

Oxygenation of **2** by either an electrophilic or radical process might yield a complex of the oxygenated steroid and the metalloenzyme represented as **4**. Analog **1** would result from some transformation of this intermediate, such as hydrolysis, and, if this transformation were reversible, then **1** could also be converted into pregnenolone by way of this common intermediate.

SCHEME 3
Proposed radical (Path A) and electrophilic (Path B) aromatic substitution mechanisms for biosynthesis of pregnenolone from *p*-tolyl analogs **1** and **2**



Since it is probable that **1** is converted into pregnenolone at a rate faster than that for the corresponding conversion of **2** (based on relative yields from incubations run under identical conditions), it may be assumed that the rate constant k_{-2} is larger than k_1 . Furthermore, the fact that **2** is converted into **1** in far greater yield than it is converted into pregnenolone (or progesterone) would indicate that k_2 is much larger than k_3 . These assumptions would mean that the interconversion of **1** and **4** is a rapid process relative to both the 20 α -oxygenation of **2** and the 22-oxygenation of **4**. Since C-22 is part of the aromatic ring, oxygenation of **4** by the same enzyme or by another enzyme associated with the cytochrome P-450 enzyme system would take place by a process similar to a free radical aromatic substitution (Path A) or an electrophilic aromatic substitution (Path B) mechanism. Concerted cleavage of the C-20,22-bond, restoration of the aromatic system, and oxidation of the metalloenzyme or some associated cofactor complexed to the oxygenated steroid at C-20 in the transition state represented by **5A** or **5B** would result in the formation of pregnenolone. Both mechanisms as drawn predict the product of the side chain fragment to be a phenol, namely *p*-cresol in the case of the *p*-tolyl analog.

It is clear from the experiments described herein that (a) the aryl side chain is capable of being cleaved enzymically to form pregnenolone; (b) the results contribute support to the idea that free hydroxylated compounds need not be true intermediates in this side chain cleavage process; and (c) while it is impossible to form a free glycol intermediate in these transformations, a C-20,22-dioxygenated steroidal intermediate or transition state is probably the species cleaved. Thus, further support has been obtained for the hypothesis previously proposed for the biosynthesis of pregnenolone from cholesterol (1). It may be worthwhile to begin to consider some consequences of this unifying concept in other areas of steroid biosynthesis (2).

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Studies on the Biosynthetic Conversion of Cholesterol into Pregnenolone: SIDE CHAIN CLEAVAGE OF SOME 20-p-TOLYL ANALOGS OF CHOLESTEROL AND 20 α -HYDROXYCHOLESTEROL

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