

Fragments Formed by the Side Chain Cleavage of a 20-Aryl Analog of 20 α -Hydroxycholesterol by Adrenal Mitochondria*

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An analog of 20 α -hydroxycholesterol, (20*R*)-20-phenyl-5-pregnene-3 β ,20-diol, which is completely substituted at C-22 was prepared with radioisotopes at various positions. The analog labeled with ^3H at C-7 and ^{14}C at C-4 and C-21 was converted into radioactive pregnenolone by an enzyme preparation derived from adrenal mitochondria. Cleavage of the phenyl analog labeled with ^3H in the aromatic ring by the same enzyme preparation led to the formation of [^3H]phenol. Using the substrate doubly labeled with ^{14}C at C-4 and ^3H in the aromatic ring, it appeared that the products of the reactions, pregnenolone and phenol, were formed in equal amounts. During incubation of the side chain labeled substrate, another labeled fragment was formed. It was identified as acetophenone, a product resulting from cleavage of the C-17,20 bond. The steroidal fragment corresponding to this C₈ ketone was traced using nuclear label analog. From its nonpolar chromatographic properties it appears to be a C-17-deoxy-C₁₉ steroid.

Previous studies (1, 2) have led us to postulate that the enzymatic reactions that lead to the biosynthesis of the steroid hormones from cholesterol¹ involve transient intermediates bound to multienzyme complexes (3). This view differs from the traditional one in that it does not consider stable, hydroxylated compounds to be obligatory intermediates in the process. Support for this thesis has come from experiments using as substrates for the cholesterol side chain cleavage enzyme analogs of the sterol which were completely substituted at C-22. Three such analogs, (20*R*)-20-(*t*-butyl)-5-pregnene-3 β ,20-diol, (20*R*)-20-(*p*-tolyl)-5-pregnene-3 β ,20-diol, and (20*S*)-20-(*p*-tolyl)-5-pregnen-3 β -ol have been shown to be converted into pregnenolone by incubation with mitochondria from adrenal glands (1, 2). Since the carbon atom corresponding to C-22 of cholesterol is completely substituted in these compounds, the involvement of stable, dioxygenated intermediates analogous to (20*R*,22*R*)-20,22-dihydroxycholesterol (4) is not possible in these transformations. Oxygenation of C-22, if necessary for cleavage, could only occur simultaneously with fission of the C-20,22 bond. Identification of the side chain cleavage product

from these analogs would help clarify the mechanism of these reactions and specifically would reveal whether or not metabolism of the substituents of the side chain to "unblock" them was required before cleavage of the C-20,22 bond occurs.

To study this problem a radioactive analog bearing the label in the side chain is required. The three compounds mentioned above did not lend themselves readily for this study. The *t*-butyl derivative was unsuitable because possible rearrangements might make the identification of the primary cleavage species difficult. A radioactive *p*-tolyl derivative is not easily accessible because no *p*-tolyl reagent in radioactive form was available inexpensively. For these reasons, we chose to prepare the 20-phenyl compound, 1, which was easily prepared from radioactive bromobenzene.

Like the other "blocked" analogs, the phenyl compound, was also converted in good yield into pregnenolone by adrenal mitochondria. If cleavage had proceeded by a radical mechanism, one of the fragments derived from the phenyl side chain could possibly have been isolated as benzene. However, no tritium was found in the easily volatilized fraction entrained by a stream of air passed through the incubation mixture. It was not surprising, therefore, that the other obvious cleavage product, radioactive phenol, was recovered from the reaction. The tritiated phenol was identified by the radiochemical homogeneity of four crystalline derivatives, its *p*-bromobenzene sulfonate, phenoxyacetic acid, its amide and its anilide.

An effort was made to establish the stoichiometry of the reaction: phenyl derivative (1) \rightarrow pregnenolone + phenol (Fig. 1) by comparing the radioactivity associated with the steroid with that found in phenol. When the experiment was corrected

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¹ Systematic names given for steroids in the text are: cholesterol, 5-cholesten-3 β -ol, 20 α -hydroxycholesterol, (20*S*)-5-cholestene-3 β ,20-diol; (20*R*,22*R*)-20,22-dihydroxycholesterol, (20*R*,22*R*)-5-cholestene-3 β ,20*R*,22*R*-triol; pregnenolone, 3 β -hydroxy-5-pregnen-20-one; progesterone, 4-pregnen-3,20-dione; androstenedione, 4-androstene-3,17-dione; testosterone, 17 β -hydroxy-4-androsten-3-one; dehydroisoandrosterone, 3 β -hydroxy-5-androsten-17-one; androstenediol, 5-androstene-3 β ,17 β -diol.

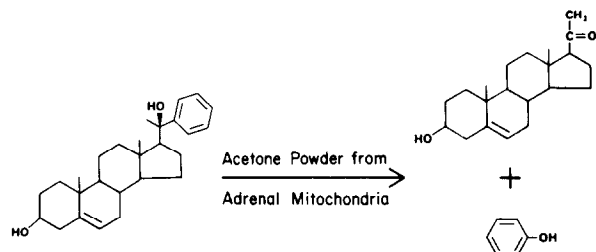


FIG. 1.

for the amount of pregnenolone (and progesterone) further metabolized to other unidentified products, the amounts of pregnenolone and phenol formed from 1 appeared to be equal.

The association of tritium with another neutral and volatile product was, on the other hand, unexpected. Its identity was established as acetophenone through its sublimable oxime. This C_8 ketone could only have been formed by cleavage between C-17 and C-20, a reaction which does not occur when cholesterol, itself, is the substrate (5, 6). When a sample of the phenyl derivative labeled at C-21 with ^{14}C was incubated with adrenal mitochondria, the acetophenone isolated contained radiocarbon, confirming that the aromatic ketone was formed by cleavage of the entire C_8 side chain.

Finally a search was made for the steroidal fragment left after the cleavage of acetophenone from the phenyl derivative. Using as substrate a sample of 1 containing tritium at C-7 of the nucleus and ^{14}C at C-21, a radioactive material less polar than androstenedione and containing solely 3H was recovered. Attempts to identify it are presently underway.

EXPERIMENTAL PROCEDURE

Melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra were taken in potassium bromide micro discs with a Perkin-Elmer model 521 grating infrared spectrophotometer equipped with dual $4\times$ reflecting beam condensers. Nuclear magnetic resonance spectra were recorded with a Varian EM-360, 60 MHz spectrometer. Mass spectra were obtained with a Dupont 21-492B mass spectrometer and a computerized data system, 21-094. Radioactive samples were counted in Packard liquid scintillation spectrometers models 3375 and 2409. The efficiency of counting for 3H was 51%, and for ^{14}C , 88%. When 3H and ^{14}C were determined together, the efficiency for 3H was 42% and the overlap into the ^{14}C channel was negligible. For ^{14}C the efficiency was 63% and the overlap into the 3H channel was 15%. The counts were corrected by the method of Okita *et al.* (7). Celite partition chromatography was done as described by Siiteri (8). All solvents were distilled prior to use. Bromobenzene (Eastman Organic Chemicals) was fractionally distilled twice through a Vigreux column and the fraction that boiled at 154.4° (uncorrected) was used for the preparation of the Grignard reagent.

Radioactive Tracers—Radioactive tracers were purchased from New England Nuclear Corp., [7α - 3H]pregnenolone, (25 Ci/mmol) and [4 - ^{14}C]pregnenolone, (51 Ci/mol) were purified by chromatography on celite in system C-1 (Table I) where pregnenolone is eluted in approximately the 6th holdback volume. [21 - ^{14}C]pregnenolone (59 Ci/mol) was purchased as the crude reaction product and was consequently purified more rigorously than were the other tracers. The radioactive material was acetylated and chromatographed on an alumina column (Woelm-neutral 3% H_2O) where it was eluted with a solution of petroleum ether in benzene (3/7). The fractions were combined, evaporated, and chromatographed on celite in system C-3 where pregnenolone acetate is eluted in the 3rd to 4th holdback volume. After saponification, the tracer was chromatographed on a silica gel (VWR Scientific) column. The chromatogram was developed with solutions of increasing amounts of benzene in petroleum ether and then ethyl acetate in benzene. The radioactive pregnenolone was eluted in benzene containing 5% ethyl acetate. Finally, the tracer was rechromatographed on celite in system C-1. The radiochemical ho-

TABLE I

Chromatography systems

In system C-2 each solvent was saturated with the other. C, column-celite partition system; T, thin layer-Silica Gel GF.

| | |
|-----|--|
| C-1 | Isooctane/methanol/water (10/9/1) |
| C-2 | Heptane/methyl cellulolve |
| C-3 | Methanol/ <i>n</i> -propyl alcohol/ H_2O /toluene/isooctane (4/0.75/2/2/2) (reverse phase) |
| T-1 | benzene/ethyl acetate (10/1) |
| T-2 | benzene/ethyl acetate (1/1) |
| T-3 | benzene/ethyl acetate (3/1) |

mogeneity of these purified tracers was established by recrystallization to constant specific activity with carrier pregnenolone using the criteria previously described (9). [U - 3H]Bromobenzene (specific activity, 19 Ci/mol) was purchased from New England Nuclear and chromatographed by preparative gas-liquid chromatography by the manufacturer just prior to shipment. It was used without further purification.

Preparation of (20*R*)-20-(Phenyl)-5-pregnen-3 β ,20-diol, 1—The phenyl analog, 1, was prepared by treating pregnenolone acetate with phenyl lithium using the same technique as that employed for the preparation of the tolyl derivative (2). Pregnenolone acetate (10 g) was added to phenyl lithium in ether and, after refluxing the mixture overnight, ice and water were added. The gummy material that separated was chromatographed on a 200-g silica gel column. After washing the column with 1 liter of a benzene/heptane (8/2) mixture, 1 liter of benzene and 1 liter of benzene containing 1% ethyl acetate, the product was eluted with benzene containing 2.5% ethyl acetate. It was crystallized from aqueous ethanol, (m.p. 234 – 238°). The compound was characterized by its infrared, nuclear magnetic resonance and mass spectra; infrared spectrum (micro KBr) 3575 (sharp, 20α -OH), 3420 (broad, 3β -OH), 1495 (aromatic ring), 1060 (Δ^4 - 3β -OH), 705 (aromatic ring); nuclear magnetic resonance was determined on the 3β -acetoxy derivative in CD_3Cl , (tetramethylsilane): δ 7.27 ppm (m, narrow, 5H, -phenyl), 5.32 (m, narrow, 1, H-6, vinyl), 4.50 (m, broad, 1, H-3 α), 2.02 (s, 3, 3β -acetate), 1.65 (s, 3, H-21), 1.03 (s, 3, H-19), 0.93 (s, 3, H-18); mass spectrum (70 ev): low resolution 394 (0.3, parent), 376 (100, base, M- H_2O), 361 (6.5, M- H_2O - CH_3), 358 (7.5, M-(2) H_2O), 343 (24.8, M-(2) H_2O - CH_3), 317 (2.7, M- C_6H_5), 256 (9.9, M- C_6H_5O - H_2O), 121 (23.3, C_6H_5O side chain).

Preparation of Radioactive (20*R*)-20-(Phenyl)-5-pregnen-3 β ,20-diol, 1—The radioactive aromatic substrates used in this study were prepared by the procedure previously described for the *p*-tolyl analog (2). The nuclear-tritiated phenyl analog, 1a, was synthesized from 1 mCi of [7α - 3H]pregnenolone. The ^{14}C -labeled phenyl analog, 1b, having the isotope at C-4 was synthesized from 20 μ Ci of [4 - ^{14}C]pregnenolone and the ^{14}C -labeled phenyl analog, 1c, having radiocarbon at C-21 was synthesized from 100 μ Ci of [21 - ^{14}C]pregnenolone. The analog labeled in the aromatic ring, the [$benzene$ - U - 3H]phenyl compound, 1d, was synthesized from 5 mCi of bromo[U - 3H]benzene. Before purification, the side chain-labeled analog, 1d, was dissolved in benzene and evaporated under vacuum repeatedly to remove volatile radioactive contaminants.

The above tracers were purified by the procedure described for (20*R*)-20-(*p*-tolyl)-5-[7α - 3H]pregnen-3 β ,20-diol (2). In brief, the residues were chromatographed on a silica gel column and this was followed by chromatography on a celite column in system C-2. The products were acetylated, the acetates chromatographed on celite in system C-3 and then rechromatographed on a column of alumina (3% H_2O). Thereafter, they were saponified and chromatographed on celite in system C-1. To confirm their radiochemical purity, a portion of these materials were combined with unlabeled carrier and crystallized. The data in Table II show that the initial specific activities of these mixtures were maintained through three crystallizations. This evidence was taken as proof of radiochemical homogeneity.

Incubation Conditions—The details of these procedures have previously been described (10). Bovine adrenals were obtained fresh from the slaughter house and brought to the laboratory on ice. The tissue was defatted, demedullated, homogenized and the mitochondrial fraction isolated by differential centrifugation. The mitochondrial pellet was lyophilized and from this dried residue an acetone powder was prepared. For use as an enzyme source, approximately 500 mg of acetone powder was suspended with sonication in 10 ml of 0.1 M tris

TABLE II
Crystallization data of radioactive (20R)-20-phenyl-5-pregnene-38,20-diol

| Isotope and Position | Fraction ^a | Solvent ^b | mg | Amount cpm | Specific Activity cpm/mg |
|--|-----------------------|----------------------|-------|---------------|-----------------------------|
| Compound 1a (7- ³ H) | X-O | | 0.330 | 773 | 2350 |
| | X-1 | B | 0.180 | 414 | 2300 |
| | ML-1 | | 0.396 | 932 | 2350 |
| | X-2 | B-H | 0.545 | 1348 | 2470 |
| | ML-2 | | 0.483 | 1188 | 2450 |
| | X-3 | A | 0.494 | 1197 | 2420 |
| | ML-3 | | 0.241 | 558 | 2310 |
| Compound 1b (4- ¹⁴ C) | X-O | | 0.694 | 910 | 1310 |
| | X-1 | B | 0.846 | 1093 | 1290 |
| | ML-1 | | 0.996 | 1300 | 1310 |
| | X-2 | A | 0.828 | 1103 | 1330 |
| | ML-II | | 0.340 | 451 | 1330 |
| Compound 1c (21- ¹⁴ C) | X-O | | 0.162 | 332 | 2050 |
| | X-1 | B | 0.249 | 517 | 2080 |
| | ML-1 | | 0.438 | 888 | 2030 |
| | X-2 | B-H | 0.628 | 1338 | 2130 |
| | ML-2 | | 0.254 | 519 | 2050 |
| | X-3 | A | 0.513 | 1020 | 1990 |
| | ML-3 | | 0.168 | 357 | 2120 |
| Compound 1d (U-benzene- ³ H) | X-O | | 0.287 | 599 | 2090 |
| | X-1 | B | 0.247 | 519 | 2100 |
| | ML-1 | | 0.387 | 808 | 2090 |
| | X-2 | B-H | 0.540 | 1172 | 2170 |
| | ML-2 | | 0.624 | 1370 | 2200 |
| | X-3 | A | 0.516 | 1080 | 2100 |
| | ML-3 | | 0.279 | 602 | 2160 |

^aX-n, crystalline product from the nth crystallization. ML-n residue left in the mother liquor from the nth crystallization. X-O, residue before crystallization. ^bA=acetone, B=benzene, E=diethyl ether, H=hexane, I=isooctane, M=methanol, P=petroleum ether (30-60°), W=water.

(hydroxymethyl) aminomethane (Tris) hydrochloride buffer, pH 7.4. The incubation mixture (final volume, 5 ml), consisted of an aliquot of the acetone powder extract and a TPNH generating system consisting of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and TPN. The steroid substrates were solubilized by dissolving them in an acetone-Tween 80 mixture (0.5 ml/50 µg) and then evaporating the acetone in a stream of N₂. Tris buffer, 1 ml, was added and the resulting suspension was sonicated to disperse the substrate. In order to aid in the solubilization of the relatively large amounts of substrates used in Experiments 2 to 5, 0.1 ml of propylene glycol was added prior to the addition of the buffer. In Experiments 3 to 5, additional amounts of the acetone powder extract and TPNH generating system were added after 30 min of incubation in order to increase yield of the cleavage products. In these experiments, the final volume of each incubation mixture was 6.0 ml.

Experiment 1: Incubation of [7-³H]phenyl Analog, 1a, with Adrenal Mitochondria—The [7-³H]phenyl analog, 1a, 1.03 × 10⁶ cpm, 14.5 ng was incubated for 30 min at 37° with an acetone powder extract prepared from bovine adrenal mitochondria containing 33 mg of protein and a TPNH generating system. The reaction was stopped by the addition of 25 ml of methanol containing 24.9 mg of carrier pregnenolone and several unlabeled C₁₉ steroids (see below). The precipitate was removed by filtration and the filtrate was evaporated to dryness. The residue was chromatographed on celite in system C-1 from which pregnenolone is eluted in the 5th to 6th holdback volume. The fractions containing pregnenolone were combined, evaporated, and acetylated with 0.5 ml of acetic anhydride in 1.0 ml of pyridine at room temperature overnight. The solvents were evaporated and the resulting pregnenolone acetate was chromatographed on celite in system C-3, from which it is eluted in the 3rd to 4th holdback volume. Those fractions containing pregnenolone acetate were combined, evaporated to dryness and the residues recrystallized from several different solvents. The data in Table III demonstrate that the specific activity throughout these recrystallizations remained constant. The yield of pregnenolone calculated from the specific activity of the product and the mass of carrier added was 53,200 cpm or a yield of 5.2%.

The following C₁₉ steroids were added at the end of the incubation as carriers: androstenedione, testosterone, dehydroisoandrosterone, and

5-androstene-3β,17β-diol. Each was reisolated by appropriate chromatographic techniques. After one crystallization the carrier steroids were found to be devoid of radioactivity.

Experiment 2: Incubation of [benzene-U-³H]Phenyl Analog, 1d, with Adrenal Mitochondria—[benzene-U-³H]Phenyl analog, 1d, 4 × 10⁶ cpm, 74 µg, was incubated at 38° for 80 min in a final volume of 5.5 ml with a TPNH generating system and an acetone powder extract prepared from bovine adrenal mitochondria (25 mg of protein). The steroid substrate was solubilized with Tween 80 and propylene glycol as described above. The reaction mixture was shaken in a 25 ml, two-necked round bottom flask with ground glass fittings into one of which an air stream slowly flowed. The exit port was connected to a glass tube (150 × 3 mm inside diameter) the other end of which was submerged in 5 ml of isooctane kept at -30°. With this technique, small amounts of benzene and other similar low boiling hydrocarbons, if formed during the incubation, are quantitatively trapped in the isooctane. At the end of the incubation, a 1-ml aliquot of the isooctane was counted in the scintillation counter and found to be devoid of any radioactivity.

Identification of Phenol—The incubation medium was then steam distilled and 50,000 counts of ³H were found in the distillate. One-quarter of the distillate, 12,500 cpm, was partitioned between 5 ml of 2 N NaOH and 10 ml of ether. The alkali extracted 8,000 cpm of tritium. Carrier phenol (112 mg) and sufficient NaOH to make the final concentration of base 33% were added. The mixture was treated with 1 g of chloroacetic acid and the solution was heated in a boiling water bath for 1 hour. After cooling, the reaction mixture was made acid to congo red paper with concentrated HCl and then extracted twice with 2 volumes of ether. The ether solution was washed twice with 2 volumes of a 5% solution of NaHCO₃. The bicarbonate extract was acidified and the acidic product was extracted into ether. The ethereal solution was dried over anhydrous Na₂SO₄ and evaporated to dryness under vacuum. The resulting phenoxyacetic acid was recrystallized several times to constant specific activity. The recrystallization data are shown in Table IV. The product was then converted into its anilide as previously described (5). This derivative was purified by chromatography on thin layer chromatography in system T-1 and recrystallized. Its specific activity was identical with that of the free acid (see Table IV). The results indicate that, of the 12,500 cpm of ³H

TABLE III
Crystallization data of pregnenolone and progesterone^a

| Experiment and Compound | Fraction | Solvent | mg | Amount | | Specific Activity | |
|--------------------------------------|------------------|---------|-------|--------------------|---------------------|-----------------------|-----------------------|
| | | | | ³ H cpm | ¹⁴ C cpm | ³ H cpm/mg | ¹⁴ C cpm |
| Experiment 1 Pregnenolone Acetate | X-1 | M | 0.391 | 695 | | 1780 | |
| | ML-1 | | 0.270 | 534 | | 1980 | |
| | X-2 | M-F | 0.495 | 924 | | 1870 | |
| | ML-2 | | 0.385 | 708 | | 1840 | |
| | X-3 | A-P | 0.358 | 672 | | 1870 | |
| | ML-3 | | 0.927 | 1744 | | 1880 | |
| Experiment 3 Pregnenolone Acetate | X-1 | A-M | 0.617 | | 76 | | 124 (44) ^b |
| | ML-1 | | 0.734 | | 143 | | 194 (69) |
| | X-2 | D-1 | 0.651 | | 75 | | 116 (42) |
| | ML-2 | | 0.170 | | 24 | | 142 (51) |
| | X-3 ^c | M | 0.632 | | 85 | | 135 (43) |
| | ML-3 | | 0.724 | | 86 | | 119 (38) |
| Pregnenolone | X-4 | A-P | 0.679 | | 89 | | 132 (42) |
| | ML-4 | | 0.620 | | 83 | | 134 (42) |
| Progesterone | X-1 | A-P | 0.953 | | 61 | | 64 |
| | ML-1 | | 0.881 | | 65 | | 74 |
| | X-2 | A | 0.717 | | 46 | | 64 |
| | ML-2 | | 0.861 | | 61 | | 70 |
| | X-3 | M | 0.994 | | 62 | | 62 |
| | ML-3 | | 0.330 | | 21 | | 62 |
| Experiment 5 Pregnenolone Acetate | X-1 | M | 0.237 | 34 | 8 | 141 | 34 |
| | ML-1 | | 0.395 | 116 | 23 | 294 | 58 |
| | X-2 | B | 0.311 | 43 | 10 | 138 | 34 |
| | ML-2 | | 0.334 | 59 | 14 | 178 | 42 |
| | X-3 | B-H | 0.674 | 93 | 23 | 138 | 35 |
| | ML-3 | | 0.283 | 40 | 10 | 143 | 36 |
| | X-4 | A-M | 0.277 | 38 | 10 | 137 | 35 |
| | ML-4 | | 0.239 | 33 | 8 | 139 | 34 |

^a See legend to Table II for an explanation of the symbols. ^b Data in the brackets are cpm/ μ mole. ^c Prior to the third crystallization, X-2 was saponified.

TABLE IV
Crystallization data of side chain fragments^a

| Experiment and Compound | Fraction | Solvent | mg | Amount | | Specific Activity | |
|--|------------------|---------|-------|--------------------|---------------------|-----------------------|------------------------|
| | | | | ³ H cpm | ¹⁴ C cpm | ³ H cpm/mg | ¹⁴ C cpm/mg |
| Experiment 2 Phenoxyacetic Acid | X-1 | A-P | 3.35 | 105 | | 31 (4.7) ^b | |
| | ML-1 | | 2.06 | 74 | | 36 (5.5) | |
| | X-2 | M-W | 1.91 | 64 | | 34 (5.2) | |
| | ML-2 | | 0.55 | 19 | | 35 (5.3) | |
| | X-3 | A | 0.74 | 24 | | 32 (4.9) | |
| | ML-3 | | 0.64 | 22 | | 34 (5.2) | |
| Phenoxyacetanilide | X-4 ^c | A-P | 0.297 | 7 | | 24 (5.4) | |
| Phenoxyacetamide | X-1 | A-P | 1.39 | 26 | | 19 | |
| | ML-1 | | 0.53 | 14 | | 26 | |
| | X-2 | A-P | 1.05 | 19 | | 18 | |
| | ML-2 | | 1.3 | 27 | | 21 | |
| | X-3 ^d | A-P | 1.18 | 20 | | 17 | |
| | ML-3 | | 0.68 | 17 | | 25 | |
| Acetophenone oxime | X-4 | A-P | 1.05 | 19 | | 18 | |
| | X-O | | 7.60 | 179 | | 24 | |
| | X-1 | A-P | 6.58 | 135 | | 21 | |
| | ML-1 | | 6.67 | 132 | | 20 | |
| | X-2 | A-P | 5.67 | 129 | | 23 | |
| | X-3 | H | 4.99 | 106 | | 21 | |
| Experiment 3 Phenyl p-bromophenyl sulphonate | ML-3 | | 3.58 | 81 | | 23 | |
| | X-4 ^e | A-P | 2.50 | 56 | | 22 | |
| | X-5 ^f | A-P | 1.55 | 34 | | 22 | |
| | X-1 | E-P | 7.52 | 80 | | 11 | |
| | X-2 | A-I | 9.46 | 103 | | 11 | |
| | ML-2 | | 2.62 | 45 | | 17 | |
| Acetophenone oxime | X-3 | I | 9.31 | 100 | | 11 | |
| | ML-3 | | 1.47 | 16 | | 11 | |
| | X-1 | H | 4.3 | 63 | | 15 | |
| | ML-1 | | 6.5 | 102 | | 16 | |
| | X-2 | P | 7.0 | 100 | | 14 | |
| | ML-2 | | 2.7 | 40 | | 15 | |
| Experiment 5 Acetophenone oxime | X-1 | A-P | 2.27 | | 34 | | 15 |
| | ML-1 | | 6.99 | | 107 | | 15 |
| | X-2 | H | 4.01 | | 62 | | 15 |
| | ML-2 | | 2.55 | | 40 | | 15 |

^a see legend to Table II for an explanation of the symbols, ^b data in the brackets are cpm/ μ mole, ^c the product of the third crystallization was converted into its anilide, which was chromatographed in system T-1 and then crystallized as X-4, ^d prior to the third crystallization X-2 was chromatographed in system T-2, ^e prior to the fourth crystallization X-3 was sublimed, ^f prior to the fifth crystallization X-4 was chromatographed in system T-1.

present in the aliquot of the steam distillate, 6,000 cpm were associated with phenol. This corresponds to a yield of 24,000 cpm or 0.6%. This value is uncorrected for the losses incurred during steam distillation since the carrier was added afterwards.

Further confirmation that the steam-volatile, alkaline-soluble product formed during the incubation was phenol was obtained. Another aliquot of the steam distillate containing 12,500 cpm of ^3H was treated with 200 mg of phenol and the mixture was extracted as described above. The phenol was converted into phenoxyacetic acid which was then refluxed for 15 min with 1 ml of SOCl_2 . The reaction mixture was poured into 3 ml of an ice-cold concentrated solution of ammonia. The amide was recovered by filtration and was recrystallized twice, purified by chromatography on thin layer chromatography in system T-2, and recrystallized twice again. The specific activity of the product remained constant throughout (see Table IV).

Identification of Acetophenone—In an effort to examine the radioactivity present in the neutral, steam-volatile fraction, an aliquot of the ether solution remaining after extraction with alkali was evaporated to dryness under a hot air blower. In the process 75% of the ^3H present was volatilized. A second portion of the ether solution was diluted with several milliliters of benzene and of toluene. Fractional distillation of the mixture revealed that all the radioisotope remained with the toluene after distillation of the ether, the benzene, and most of the toluene. This suggested that the radioactive component had a boiling point greater than that of toluene. In order to identify the functional group(s) present in the tritiated component, attempts were made to prepare less volatile derivatives whose formation could easily be detected by taking advantage of this property. Thus, for example, as much radioactivity was lost by volatilization under a stream of hot air after treatment with benzoyl chloride (in pyridine) as had been lost from the untreated extract. Likewise treatment with H_2O_2 was without effect. However, reaction with either hydroxylamine or semicarbazide hydrochloride resulted in retention of more than 2% of the volatile tritium. These results indicated that the volatile radioactive substance was a moderately high boiling ketone, probably acetophenone, a product that could be formed by cleavage between C-17 and C-20 of the phenyl analog. Carrier acetophenone, 200 mg, was, therefore, added to the ether extract from a 25% aliquot of the steam distillate. After extraction with NaOH solution the ether was dried over Na_2SO_4 and then carefully evaporated by distillation through a Vigreux column. To the residual liquid was added 1 ml of ethanol, 1 ml of pyridine, and 200 mg of hydroxylamine hydrochloride, and the resulting mixture was refluxed on a steam bath for 2 hours. The solvents were removed under vacuum and the residue was triturated with water. The product was filtered and recrystallized three times (Table IV). It melted at 58° (lit. 59°). The oxime remaining from the third crystallization was sublimed at 62° at less than 1 mm onto a cold finger. After crystallization, the product was purified by chromatography on thin layer chromatography in system T-1, and recrystallized once again. Throughout all of these procedures, the specific activity of the oxime remained constant (Table IV). The yield of acetophenone in this experiment was calculated to be 20,000 cpm or 0.5%.

As a control, the [*benzene- U - ^3H*]phenyl analog was incubated with a sample of mitochondria that previously had been heated at 90° . The steam distillate from this incubation contained less than 1100 cpm of tritium.

Experiment 3: Incubation of Nuclear-, 1b, and Side Chain-, 1d, labeled Phenyl Analog with Adrenal Mitochondria—In an effort to establish the stoichiometry of the side chain cleavage reaction, [$4\text{-}^{14}\text{C}$]phenyl analog (1b), 798,000 cpm (4.4 μg) was mixed with 670,000 cpm (15 μg) of [*benzene- U - ^3H*]phenyl analog (1d) and incubated with adrenal mitochondria (45 mg of protein) as described above. After 30 min of incubation, another sample of the mitochondrial preparation (corresponding to 45 mg of protein) was added and after a further 30 min of incubation, 190 mg of phenol, 206 mg of acetophenone, 3.1 mg of pregnenolone, and 3.75 mg of progesterone were added. The carriers were reisolated without steam distillation. The incubation mixture was extracted with three portions of 5 ml of ether. The combined ether extracts were then extracted twice with 15 ml of 2 N NaOH. The alkaline solution was acidified and extracted two times with 15 ml of ether. The ether solution was dried over Na_2SO_4 and the ether removed by distillation. The residual phenol was distilled at 40° under vacuum. The phenol was dissolved in 0.5 ml of pyridine and treated overnight at room temperature with 300 mg of *p*-bromobenzene sulfonyl chloride. The pyridine was evaporated by a stream of nitrogen and the residue partitioned between 100 ml of ether and 15 ml of 1%

NaHCO₃ solution. The ether extract was washed twice again with 15 ml of bicarbonate solution and then with water. The solvent was removed by evaporation and the residue was chromatographed on a 20-g silica gel (Woelm) column. The column was developed with increasing amounts of benzene in heptane and finally the *p*-bromophenyl sulfonate was eluted with a 1/1 benzene/heptane solution. Those fractions containing the ester were combined and recrystallized (m.p. $114\text{--}115^\circ$, value found in literature 115.5°) to constant specific activity (Table IV). From these data it was calculated that 6,800 cpm of [^3H]phenol were enzymatically cleaved from the substrate.

The original ether solution remaining after the alkali extraction was washed with H_2O until neutral. The solvent was evaporated and the residue chromatographed on a 30-g silica gel column. Acetophenone was eluted with benzene and the labeled steroids were eluted with a methanol solution containing 5% methanol in benzene. The fractions containing acetophenone were combined and the benzene was removed by careful distillation. The residual liquid was then distilled in a micro-distillation apparatus at 40° at a pressure of less than 1 mm Hg. The distillate was converted into the oxime by refluxing for 2 hours with 200 mg of hydroxylamine hydrochloride, 0.5 ml of pyridine, and 0.5 ml of ethanol. Acetophenone oxime was sublimed (at 60° and less than 1 mm Hg). The resulting crystalline material was recrystallized to constant specific activity (Table IV) from which it was determined that 3360 cpm of tritiated acetophenone had been synthesized.

The steroidal residue was chromatographed on celite in system C-1 where progesterone is eluted in the 3rd to 4th holdback volume and pregnenolone in the 5th to 6th holdback volume. The fractions containing pregnenolone were evaporated and chromatographed by thin layer chromatography in system T-3. A small strip of the plate was sprayed with rhodamine G (1) and then viewed under ultraviolet light. The area where pregnenolone occurred (R_f 0.3) was scraped and the steroid eluted from the silica gel with methanol. The alcoholic solution was evaporated to dryness under vacuum and the residue was acetylated. After the reagents were removed by a stream of nitrogen, the steroidal acetate was chromatographed on celite in system C-3. Those tubes containing pregnenolone acetate were combined, evaporated to dryness, and quantified (11). The recovery was 53%. Additional carrier (11.4 mg) was added and the mixture was crystallized twice. Thereafter it was saponified and the product was crystallized twice again. The specific activity of the crystalline pregnenolone with respect to ^{14}C was constant (Table III) throughout both crystallizations. The pregnenolone acetate contained 18 cpm/mg of ^3H after two crystallizations, but saponification and crystallization afforded a product that, as expected, was devoid of ^3H .

The progesterone fraction obtained by chromatography on celite in the C-1 system was rechromatographed on thin layer chromatography in system T-1. The material containing the progesterone (visualized by ultraviolet light) was eluted with methanol and quantified by its absorbance at 240 nm. The recovery was 84%. Additional carrier, 11.3 mg, was added and the mixture crystallized to constant specific activity with respect to ^{14}C (Table III). From the specific activities of the products and the percentage recoveries of the added carriers, it was estimated that a total of 1,090 cpm of progesterone and 2,980 cpm of pregnenolone were synthesized. Calculating from the total amount of C_{21} steroids recovered, 4,000 cpm, and the original $^3\text{H}/^{14}\text{C}$ ratio (0.84) for the substrate, 3,431 cpm of [^3H]phenol should have been formed. The actual number of counts of ^3H associated with phenol was 6,840 cpm, suggesting that either the reaction is not stoichiometric as pictured in Fig. 1 or that the C_{21} steroids biosynthesized, pregnenolone and progesterone, were metabolized further.

A control incubation was performed in exactly the same way except that the mitochondrial acetone powder was heated in a boiling water bath for 10 min prior to use. Upon reisolation, the phenol, acetophenone, and pregnenolone carriers were devoid of radioactivity.

Experiment 4: Incubation of [$7\alpha\text{-}^3\text{H}$]Pregnenolone with Adrenal Mitochondria—Since the amount of C-21 steroids produced was not stoichiometric with the amount of phenol formed, an effort was made to determine whether pregnenolone was metabolized to other, unidentified steroids during the reaction with adrenal mitochondria.

[$7\alpha\text{-}^3\text{H}$]Pregnenolone, 1.06×10^6 cpm and 20 μg of nonradioactive pregnenolone were incubated with 37.5 mg of adrenal mitochondrial protein (for 30 min at 37°) as previously described. At the end of the incubation, an additional 37.5 mg of mitochondrial protein and fresh TPNH generating system was added to the mixture and the incubation was continued for another 30 min. Then, 29 mg of carrier pregnenolone, and 11.5 mg of progesterone were added in 30 ml of methanol. The

precipitated protein was filtered and the alcoholic filtrate was evaporated to dryness. The residue was chromatographed on celite in system C-1. The fractions (3rd holdback volume) containing progesterone were evaporated and the product was crystallized once from acetone-petroleum ether. The specific activity of the crystalline material was found to be 6,000 cpm/mg. Thus a total of 69,191 cpm of progesterone had been formed. Pregnenolone was eluted in the 5th and 6th holdback volume and these fractions were treated in the same manner as described above. Pregnenolone was found to have a specific activity of 3630 cpm/mg, indicating that a total of 222,720 cpm were recovered. Of the 1×10^6 cpm of pregnenolone incubated, only 291,910 cpm or 29% could be accounted for as recovered pregnenolone and progesterone. The remainder of the radioactive material, 658,000 cpm, was recovered by washing the celite column with methanol. Identification of the substances associated with this radioactivity was not attempted.

Experiment 5: Incubation of [21- 14 C]Phenyl Analog, 1c, and [7- 3 H]Phenyl Analog, 1a, with Adrenal Mitochondria—(20R)-20-(phenyl)-5-[21- 14 C]pregnene-3 β ,20 diol, 1c, (0.97×10^6 cpm, 4.7 μ g) was mixed with (20R)-20-(phenyl)-5-[7- 3 H]pregnene-3 β ,20 diol, 1a, (4×10^6 cpm, 68 ng) and incubated with adrenal mitochondria acetone powder (corresponding to 32.5 mg of protein) for 30 min at 37°. At the end of the incubation period an equal quantity of acetone powder with an equal amount of the TPNH generating system was added and the incubation continued for another 30 min. Then, 27 mg of pregnenolone and 203 mg of acetophenone were added. In addition, 15- to 25-mg portions of the following unlabeled C_{19} steroids were also added: androstenedione, testosterone, dehydroisoandrosterone, and Δ^5 -androstenediol. The mixture was extracted with ether and the ether-soluble residue was chromatographed on a silica gel column (40 g) where the acetophenone carrier was eluted with a 1:8 mixture of petroleum ether in benzene. The steroids were eluted from the column with methanol. As described above the acetophenone was distilled, converted to its oxime, which was sublimed, and finally crystallized to constant specific activity with regard to 14 C (Table IV). No 3 H was present in the crystalline material but 3,435 cpm of [14 C]acetophenone was formed.

The steroidal fraction was chromatographed on celite in system C-1, where pregnenolone and androstenedione are eluted in the 5th and 6th holdback volume. These fractions were combined and evaporated to dryness. The residue was acetylated and the product was chromatographed in system C-2, in which androstenedione is found in the first few fractions and pregnenolone acetate is eluted in the 3rd to 4th holdback volume. The fractions containing pregnenolone acetate were combined and evaporated to dryness and the residue was recrystallized to constant specific activity with respect to both 3 H and 14 C (Table III). The yield of pregnenolone was 5,500 cpm of 3 H, or 0.14%.

The C_{19} steroids were recovered from the celite column, system C-1, by washing with methanol. These steroids and androstenedione were further purified by chromatography on celite and on thin layer chromatography. The purified carriers contained insignificant amounts of radioactivity. The little radioactivity they possessed was found to be from both 14 C and 3 H and these occurred in the same ratio as that of the substrate. Thus they appeared to be products whose side chains were still intact and consequently they were not further investigated. The only fractions found to contain 3 H alone were eluted at the end of the first holdback volume in the first celite column, system C-1, suggesting that the metabolite(s) was extremely nonpolar.

DISCUSSION

It is quite likely that the enzymatic conversion of the phenyl compound, 1, to pregnenolone described in this paper proceeds by the same mechanism as that which is associated with the transformation of the *p*-tolyl analog previously reported (2). In our previous paper two possible mechanisms for the side chain cleavage of these aromatic derivatives were proposed. Both mechanisms suggest the intermediacy of reactive complexes of the oxygenated steroidal species and the metalloenzyme. In one, the active oxygen species is considered to be radical in nature and in the other it is electrophilic.

Oxygenation at C-22, which in the phenyl and *p*-tolyl derivatives is part of the aromatic ring, would occur by a mechanism involving either a free radical aromatic substitution or an electrophilic aromatic substitution. In either case,

the species cleaved is a C-20,22 dioxygenated steroidal intermediate which by fission of the C-20,22 bond in concert with 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 would result in the formation of pregnenolone. In one mechanism, the transient intermediate can be considered to be a radical and in the other it may be a carbonium ion although the distinction between these two extremes, as previously pointed out (1), may be artificial in species complexed with an iron-containing enzyme. In any case, neither mechanism proposes that the true intermediates are stable, isolable hydroxylated compounds. Both mechanisms easily rationalize the formation of a phenolic fragment.

In order to study the stoichiometry of the reaction in which pregnenolone and phenol were produced from the phenyl analog, the doubly labeled precursor (3 H in the side chain, 1d, and 14 C in the nucleus, 1b, 3 H/ 14 C = 0.84) was incubated with the adrenal mitochondrial enzyme and the amounts of pregnenolone, progesterone, and phenol formed were determined (Experiment 3). From the specific activity of the isolated carriers it could be estimated that 6,000 cpm of 3 H in phenol and 4,000 cpm of 14 C in both C_{21} steroids were produced. From this it would appear that the amounts of steroids formed did not equal the amount of phenol produced but as the results of Experiment 4 reveal pregnenolone (and probably progesterone also) undergoes extensive metabolism to other unaccounted for products under the conditions of incubation used for the phenyl compound. Only 29% of pregnenolone could be recovered when it was used as a substrate for the enzyme preparations. When all the pregnenolone is available from the very beginning of the reaction, as in Experiment 4, the amount metabolized to unidentified products would be expected to be greater than it would be when pregnenolone was the product of the cleavage reaction of the phenyl analog as it was in Experiment 3. In accord with these considerations, the results of Experiments 3 and 4 suggest that the cleavage can be represented by the equation: phenyl analog \rightarrow pregnenolone + phenol.

The isolation of acetophenone (Fig. 2) as a cleavage product from the phenyl derivative, 1, was surprising because it could only arise by cleavage of the C-17,20-bond. In the natural process where cholesterol is the substrate, rupture of this bond does not occur (5, 6). The suggestion that $C_{19}O_2$ androgens such as dehydroisoandrosterone or androstenedione could be formed from cholesterol by cleavage of all eight carbon atoms of its side chain in the form of 6-methyl-heptan-2-one (12, 13) has not been confirmed (5, 6). Apparently, however, the presence of an aromatic substitution at C-20 in the phenyl analog facilitates cleavage of the C-17,20-bond by stabilizing the incipient radical or ionic species that could, in the transition state, be generated on C-20. No such facilitation would be expected when the substitution on C-20 is alkyl (isohexyl) as it is in cholesterol. Whether a second oxygenation at C-17 is required for scission of the C-17,20-bond in the phenyl compound is not

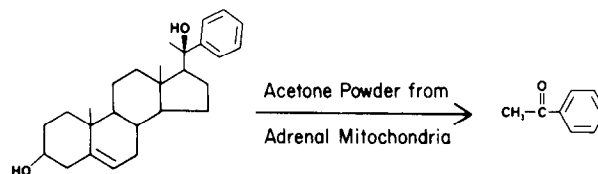


FIG. 2.

known; identification of the steroidal fragment of this reaction might help answer this uncertainty. As mentioned above, the tritiated fragment produced when the [7α - ^3H]phenyl analog was incubated with an adrenal mitochondrial preparation has chromatographic properties that are less polar than those of the C_{19}O_2 compounds, dehydroisoandrosterone, androstenedione, or testosterone. When these compounds were used as carriers, they were all reisolated devoid of radioactivity, adding support to the suggestion that the steroidal fragment may be a C_{19}O compound.

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