

Identification of 17-Methyl-18-norandrosta-5,13(17)-dien-3 β -ol, the C₁₉ Fragment Formed by Adrenal Side Chain Cleavage of a 20-Aryl Analog of (20S)-20-Hydroxycholesterol*

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Incubation of (20R)-20-phenyl-5-pregnene-3 β ,20-diol, an aromatic analog of (20S)-20-hydroxycholesterol, with an adrenal mitochondrial preparation leads to the formation of four compounds: pregnenolone, phenol, a C₈ ketone, acetophenone, and a nonpolar C₁₉ compound. This latter compound has now been identified by reverse isotope dilution analysis and by gas chromatography/mass spectrometry as 17-methyl-18-norandrosta-5,13(17)-dien-3 β -ol. From these results it is evident that enzymatic fission of the C-17,20 bond of this synthetic derivative occurs. On the other hand, when (20S)-20-hydroxy[21-¹⁴C]cholesterol was used as substrate, the analogous cleavage did not take place. Thus, substitution of an aromatic group on C-20 facilitates side chain cleavage between that carbon atom and the nucleus whereas neither of the naturally occurring precursors, cholesterol or its 20-hydroxylated counterpart, are metabolized to a C₈ fragment.

In a previous paper (1), we reported that a 20-aryl analog of (20S)-20-hydroxycholesterol would serve as a substrate for the side chain cleavage enzyme system present in mitochondria obtained from the cortex of bovine adrenals. The precursor, (20R)-20-phenyl-5-pregnene-3 β ,20-diol, labeled at C-7 with tritium was converted by an enzyme preparation derived from these mitochondria into radioactive pregnenolone. When the substrate was doubly labeled with ¹⁴C at C-4 of the steroid nucleus and with ³H in the aromatic ring, three other products of the reaction were detected. Two of these contained only ³H. One, phenol, was the product expected from the reaction in which pregnenolone had been formed by cleavage of the bond linking C-20 with the aryl group. The other tritiated product was acetophenone. That this C₈ ketone was formed by fission of the C-17,20 bond of the precursor was confirmed in another experiment in which [¹⁴C]acetophenone was isolated following the incubation of a doubly labeled substrate containing ¹⁴C at C-21 and ³H at C-7. The C₁₉ nuclear fragment resulting from this reaction naturally contained only ³H whose presence aided in the examination of this moiety.

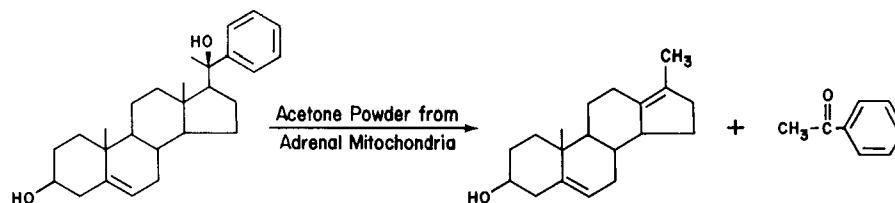
This paper describes the isolation and characterization of the C₁₉ steroidal fragment. It has been identified as 17-methyl-18-norandrosta-5,13(17)-dien-3 β -ol (*Compound 1*) (Fig. 1).

This product is probably derived by rearrangement of the carbonium ion resulting from fission between C-17 and C-20 of the phenyl analog.

This paper also describes another attempt to demonstrate that all eight carbon atoms of cholesterol can be cleaved by adrenal enzymes to yield a C₁₉ fragment and a C₈ ketone, 6-methyl-2-heptanone. Enzymatic cleavage of the C-17,20 bond of cholesterol was a subject of interest some years ago when it was reported (2, 3), that incubation of [26-¹⁴C]cholesterol with preparations of adrenals or testes led to the formation of labeled 6-methyl-2-heptanone, a C₈ ketone which presumably arose by fission of the C-17,20 bond of the sterol. Two groups of investigators (4, 5), using side chain-labeled cholesterol as precursor, failed to confirm these findings. Nevertheless, the results of the present study raised the possibility that a better precursor for this cleavage might be the 20-hydroxylated derivative of cholesterol, since it was the 20-hydroxylated derivative of the phenyl analog which, in this study, was found to be cleaved between C-17 and C-20. When (20S)-20-hydroxy[21-¹⁴C]cholesterol was incubated with an acetone powder prepared from mitochondria obtained from the cortex of bovine adrenal, no ¹⁴C-labeled 6-methyl-2-heptanone was isolated. Thus, it appears that the unusual cleavage of the phenyl analog between C-17 and C-20 occurs because the substituent on C-20 is an aromatic group. The ability of the phenyl substituent to stabilize an ionic species at C-20 undoubtedly facilitates C-17,20 fragmentation. The resulting intermediate bearing a positive charge at C-17 rearranges to yield the 17-methyl-18 norandrostadienol.

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FIG. 1. C-17,20 cleavage of (20R)-20-phenyl-5-pregnene-3 β -20-diol.

EXPERIMENTAL PROCEDURE

Melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were recorded with a Varian HA-100 spectrometer. Gas chromatography separations were performed on a Perkin-Elmer 3920 gas chromatograph. Mass spectra were obtained with a Du Pont 21-492B mass spectrometer and a computerized data system, 21-094. High pressure liquid chromatography was carried out on a Waters ALC-100 liquid chromatograph equipped with a 254 nm differential ultraviolet detector and a refractometer. Celite partition chromatography was done as described by Siiteri (6). All solvents were distilled prior to use. Radioactive samples were counted in Packard liquid scintillation spectrometer 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).

Radioactive tracers were purchased from New England Nuclear Corp. [7α - ^3H]Pregnenolone (25 Ci/mmol) and [21 - ^{14}C]pregnenolone (59 mCi/mmol) were purified as previously described (1). The nonradioactive sample of the phenyl analog, (20R)-20-phenyl-5-pregnene-3 β ,20-diol, as well as the 21 - ^{14}C and 7α - ^3H -labeled tracers of this compound were synthesized by treating pregnenolone acetate with phenyl lithium (1), using the same technique as that employed for the tolyl derivative (8). (20S)-20-Hydroxy [21 - ^{14}C]cholesterol was prepared by the reaction of [21 - ^{14}C]pregnenolone acetate with isohexyl magnesium bromide as previously described (9). The reaction product was acetylated and purified by chromatography on celite in System C-1 (Table I) where it migrates in the 3rd holdback volume. The appropriate fractions were combined, the material contained in them saponified, and the product purified by chromatography on celite in System C-2 where the tracer migrates in holdback volumes 2 and 3.

The identity and purity of all the tracers was established by reverse isotope dilution (10) and were found to be pure within experimental error. All tracers were repurified in System C-2 shortly before use in the incubation experiments. The details of the incubation procedures and the preparation of the bovine adrenal cortex mitochondrial acetone powders have been previously described (1, 11).

17-Methyl-18-norandrostadien-3 β -ols—The C-17-deoxy- C_{19} steroids, *Compounds 1, 2, and 3* (Fig. 2) were synthesized by the Bamford-Stevens elimination reaction (12). Dehydroisoandrosterone-17-tosylhydrazone was prepared by heating at reflux temperature for 6 h 200 ml of methanol containing 4 g of dehydroisoandrosterone, 2.8 g of tosylhydrazine, and 2 drops of H_2SO_4 . The reaction mixture was concentrated to 100 ml and cooled and the product which crystallized collected by filtration. It was washed with methanol and crystallized again from ether, m.p. 172–174°. The steroidal tosylhydra-

zone (2 g) was added to 50 ml of ethylene glycol in which 1 g of sodium had been dissolved. The mixture was heated at 140° for 1 h, cooled, and diluted with 150 ml of water. The precipitate was filtered and dissolved in benzene. The benzene solution was washed with water, dried over Na_2SO_4 , and evaporated to dryness yielding 0.7 g of crystalline material (60%).

Three hundred milligrams of the resulting product was chromatographed by high pressure liquid chromatography on a column (108 \times $\frac{1}{8}$ inch) of C_{18} /Porasil B (Waters Co.) with $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, (9/1) at a flow rate of 9.5 ml/min. After the first pass through the column, two poorly resolved peaks were obtained (see Fig. 3). As subsequent analysis showed, the first section of the ascending limb of the first peak contained pure Compound 1, and the end of descending limb of the last peak contained pure Compound 3. Each was collected and the remainder of the material was recycled through the chromatography column. The process of removing the material at the extremes of the eluted peaks was repeated five additional times until the middle fraction containing Compound 2 was completely purified (Fig. 3). Proper fractions were pooled and crystallized from acetonitrile. The yields of the three C_{19} steroids were: Compound 1, 22.5%; Compound 2, 7.5%; Compound 3, 37.5%. The compounds were analyzed by mass and nuclear magnetic resonance spectroscopy and the structures (Fig. 3) were assigned from these data and from what was to be expected from previous experience (12).

Compound 1: m.p. 98.5–99.5°; nuclear magnetic resonance (CDCl_3 , tetramethylsilane): δ 5.36 (m, 1, H-6, vinyl), 3.52 (m, 1, H-3 α), 1.75 (s, 3, 17- CH_3 , allylic), 0.92 ppm (s, 3, H-19); mass spectrum (30 eV): 272 (100, parent and base), 257 (18, M- CH_3), 254 (19, M- H_2O), 239 (44, M- CH_3 - H_2O), 213 (13), 199 (11), 187 (19), 171 (9), 161 (39), 159 (38), 147 (31), 145 (52), 143 (34).

Compound 2: m.p. 101–103°; nuclear magnetic resonance (CDCl_3 , tetramethylsilane): δ 5.34 (m, 2, H-6 and H-2, vinyl), 3.54 (m, 1, H-3 α), 1.04 (s, 3, H-19), 0.97 ppm (d, 3, 17- CH_3); (CDCl_3 , with EuCDPM added): δ 5.55 (m, 1, H-6, vinyl), 5.41 (m, 1, H-12, vinyl), 5.22 (m, 1, H-3 α), 3.44 (m, 2, H-4), 1.26 (s, 3, H-19), 1.06 ppm (d, 3, 17- CH_3); mass spectrum (30 eV): 272 (100, parent and base), 257 (14, M- CH_3), 254 (32, M- H_2O), 239 (45, M- CH_3 - H_2O), 213 (13), 197 (12), 149 (18), 145 (22), 143 (16).

Compound 3: m.p. 74–76°, nuclear magnetic resonance (CDCl_3 , tetramethylsilane): δ 5.40 (m, 1, H-6, vinyl), 3.54 (m, 1, H-3 α), 0.99 (d, 3, 17- CH_3), 0.94 ppm (s, 3, H-19); mass spectrum (30 eV): 272 (100, parent and base), 257 (16, M- CH_3), 254 (42, M- H_2O), 239 (91, M- CH_3 - H_2O), 213 (37), 199 (28), 187 (12), 171 (14), 161 (19), 159 (25), 147 (38), 145 (39), 143 (46).

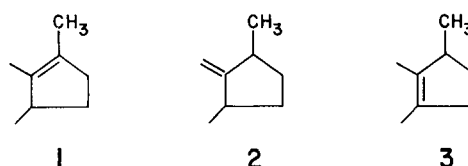
NMR data indicated that 1 has one vinyl hydrogen atom and an allylic methyl group and therefore it was assigned the structure 17-methyl-18-norandrosta-5,13(17)-dien-3 β -ol. The NMR spectra of 2 indicated that it has two vinyl hydrogen atoms and a methyl group vicinal to one hydrogen atom. These data are consistent with the structure 17-methyl-18-norandrosta-5,12-dien-3 β -ol. Compound 3 also has a methyl group vicinal to one hydrogen atom but has only one vinyl hydrogen atom. Based upon this evidence, Compound 3 was assigned

TABLE I
Chromatography Systems

C-1 ^a	methanol/ <i>n</i> -propyl alcohol/ H_2O /toluene/isooctane (4/1/1.4/2/2) (reverse phase)
C-2	isooctane/methanol/ H_2O (10/9/1)
C-3	methanol/ H_2O /toluene/isooctane (4/2/2/2) (reverse phase)
C-4	methanol/ <i>n</i> -propyl alcohol/ H_2O /toluene/isooctane (4/0.75/2/2/2) (reverse phase)
T-1 ^b	benzene/ethyl acetate (5/1)
T-2	methylenechloride/acetone (9/1)

^a C, column-celite partition system.

^b T, thin layer-silica gel.

FIG. 2. Partial structures of three C_{19}O products formed by treatment of dehydroisoandrosterone tosylhydrazone with sodium in ethylene glycol (the Bamford-Stevens elimination reaction).

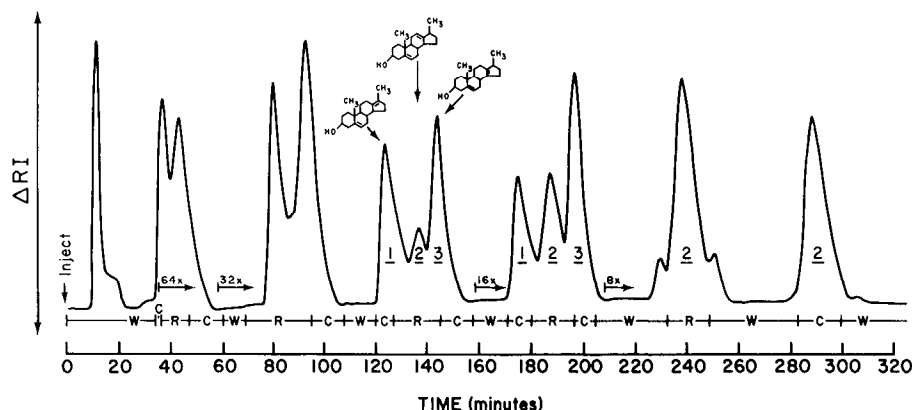


Fig. 3. Recycling high pressure liquid chromatography of the products of the Bamford-Stevens elimination reaction. For details see text. ΔRI = change in refractive index. Values followed by x represent the attenuation of the refractive index detector. W, effluent sent to waste; C, effluent collected; R, effluent recycled.

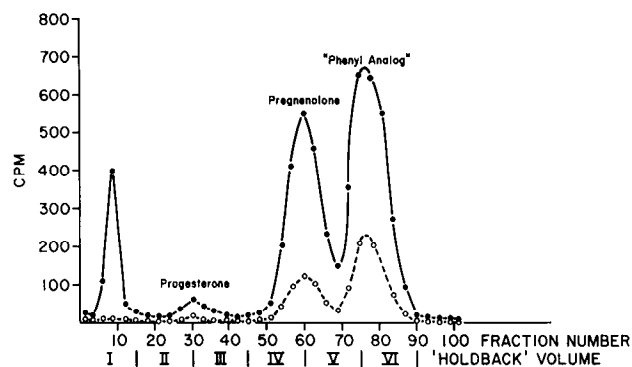


Fig. 4. Chromatographic analysis of the extract from the incubation of (20R)-20-phenyl-5-[7 α - 3H , 21- ^{14}C]-pregnene-3 β -20-diol with the adrenal mitochondrial extract. For details see text. \bullet , 3H ; \circ , ^{14}C .

the structure 17-methyl-18-norandrost-5,13-dien-3 β -ol. The mass spectra of the three substances were consistent with these assignments.

Incubation of Phenyl Analog—The [7 α - 3H , 21- ^{14}C]phenyl analog, (5×10^5 cpm of ^{14}C and 2×10^6 cpm of 3H) was incubated with a TPNH generating system and an acetone powder (32 mg of protein) isolated from bovine adrenal mitochondria. The incubation was allowed to proceed in air for 30 min at 37° and was then terminated by the addition of 5 volumes of methanol. The proteins were removed by filtration and the solvents were evaporated under vacuum. The residue was chromatographed on celite on System C-2 where a nonpolar peak containing 3H alone was eluted at the end of the 1st holdback volume (Fig. 4). The fractions containing this nonpolar, radioactive material (22,000 cpm of 3H) were pooled and the solvents evaporated. An aliquot containing 5,000 cpm of tritium was diluted with 10 mg of 5,16-androstadien-3 β -ol and the mixture was recrystallized once from methanol and then twice from acetone/petroleum ether. The product of the last crystallization contained the bulk of the radioactivity and was rechromatographed on celite in System C-2, then again on silica gel thin layer plates in System T-1. On both of these systems, the radioactivity and the carrier co-chromatographed. By all these procedures, the radioactive component appeared to be identical with the $\Delta^{16}C_{19}$ steroid. Ordinarily, these criteria of radiochemical homogeneity would have sufficed as proof that the radioactive tracer and the added carrier, 5,16-androstadien-3 β -ol, were identical. However, as subsequent work showed, the radioactive product was, in fact, 1. It is evident that the chromatographic and recrystallization procedures used up to this point in the purification process were not adequate to separate the radioactive component from the added Δ^{16} carrier.

Another chromatographic analysis was carried out, this time on silver nitrate-impregnated silica gel plates in System T-2. In this system, the unlabeled $\Delta^{16}C_{19}$ steroid migrated with an R_F = 0.2 and separated from the tritiated material whose R_F was 0.45. Moreover, upon high pressure chromatography on a C_{18} /Corasil column ($36 \times \frac{1}{8}$ inch) (Waters Co.) in the solvent system, acetonitrile/ H_2O (7/3) at a flow rate of 0.5 ml/min, further separation was achieved. The Δ^{16} carrier was eluted with a retention time of 11.2 min (using the refractive index detector) while the radioactive unknown had a reten-

tion time of 11.8 min. When fractions were collected every 15 s, the bulk of the radioactivity could be physically separated from the carrier.

Because of the close similarity in chromatographic properties, these results suggested that the radioactive product was probably also a monooxygenated C_{19} steroid. The relatively rapid migration of the unknown on silver nitrate-impregnated plates indicated either that ring D of the substance was completely saturated or that, if it were an olefin, the double bond was more highly substituted than one between C-16 and C-17. The radioactive material separated from the ring D saturated steroid, 5-androst-3 β -ol (R_F = 0.54) on the silver nitrate-impregnated plates in System T-2. This indicated that the product probably was a positional isomer of 5,16-androstadien-3 β -ol having a double bond at some location other than between C-16 and C-17. The formation of a carbonium ion at C-17 accompanying the cleavage of the C-17,20 bond when acetophenone was formed could easily be followed by rearrangement with the migration of the C-18 methyl group to C-17 and formation of a double bond between C-13 and C-17 (Compound 1), between C-13 and C-14 (Compound 2), or between C-12 and C-13 (Compound 3).

Of the three products isolated from the Bamford-Stevens elimination reaction on the tosylhydrazone (Fig. 2), the authentic $\Delta^{13, (17)}$ isomer, 1, had the same chromatographic properties on the C_{18} /Corasil column and also on the silver nitrate thin layer plate as that of the radioactive product from the incubation. One thousand counts per min of the biosynthetic product was combined with 100 μg of Compound 1 and the mixture chromatographed on the C_{18} /Corasil column as previously described. Fractions of 0.5 ml were collected and the radioactivity and the amount of carrier was determined in each fraction. Over 70% of the radioactivity was found to migrate precisely with the carrier. A small peak of 3H was also found traveling close to the front of the column and because it was so polar it was presumed to be an autooxidative product of the main peak. One hundred milligrams of Compound 1 were combined with the radioactive product (1000 cpm of tritium) isolated on celite in System C-2 and the mixture was chromatographed by high pressure liquid chromatography on C_{18} /Porasil B in acetonitrile/ H_2O , at a flow rate of 9.6 ml/min. After the first pass, the appropriate material was recirculated through the column three additional times to increase the resolution. The fractions containing Compound 1 were combined and the specific activity of the recovered crystalline material was found to be 7.2 cpm/mg. Thus, 72% of the radioactive product had co-chromatographed with the $\Delta^{13, (17)}$ isomer (Compound 1) in this specific system. This mixture of synthetic 1 with the radioactive product was recrystallized three times, first with acetonitrile/ H_2O , then from acetonitrile alone, and finally from acetone/petroleum ether. The specific activity of the crystalline products were all identical, 7.2 cpm/mg.

Another incubation was performed exactly as described for the previous experiment with the exception that only [7 α - 3H]phenyl analog, 10×10^6 cpm, was incubated with the acetone powder. After incubation, 5.7 mg of Compound 1 was added to the mixture in 100 ml of methanol. The proteins were removed by filtration, the filtrate evaporated to dryness, and the residue chromatographed on celite first in System C-2 and then again in System C-3 where the carrier was eluted in holdback volumes 4 and 5. Those fractions containing the C_{19} steroid were combined and evaporated and the amount of radioactivity present in those fractions was found to be 114,800 cpm. The steroidal residue was crystallized several times. After the first two crystalliza-

TABLE II
Crystallization data

Sample	Fraction ^a	Solvent ^b	Amount		Specific activity
			mg	cpm	cpm/mg
17-Methyl-18-norandrosta-5,13(17)-diene-3 β -ol ^c	X-1	An-W	0.104	2,323	22,300
	ML-1		0.324	13,930	43,000
	X-2	A-P	0.104	1,935	18,600
	ML-2		0.125	4,155	33,300
	X-3	An-W	0.150	2,343	15,600
	ML-3		0.058	1,283	22,000
	X-4	An	0.077	1,191	15,500
	ML-4		0.030	473	15,700
	X-5	An-W	0.054	788	14,600
	ML-5		0.056	854	15,200
Pregnenolone acetate ^d	X-0		0.930	34,954	37,570
	X-1	A-P	0.102	3,636	35,650
	ML-1		0.114	4,389	38,530
	X-2	M-W	0.432	16,466	38,160
	ML-2		0.384	13,986	36,460
	X-3	M	0.373	13,721	36,800
	ML-3		0.365	14,735	40,350
	X-4	A-P	0.415	15,386	37,050
	ML-4		0.363	14,479	39,850
Semicarbazone of 6-methyl-2-heptanone ^d	X-0		0.129	1	9
	X-1	An-W	0.994	6	6
	X-2	A-P	0.641	2	3

^a X-0, residue before crystallization; X-*n*, crystalline product from the *n*th crystallization; ML-*n*, residue left in the mother liquor from the *n*th crystallization.

^b An, acetonitrile; W, water; A, acetone; P, petroleum ether (30–60°);

M, methanol.

^c Isolated from the incubation of (20*R*)-20-phenyl-5-pregnene-3 β ,20-diol.

^d Isolated from the incubation of (20*S*)-20-hydroxycholesterol.

tions the specific activity of the crystals and the residues in the mother liquors were constant (Table II).

Reliance upon radiochemical homogeneity as proof of identification is, at best, risky particularly with nonpolar compounds such as those involved in this study. Such compounds are difficult to separate chromatographically and are known to co-crystallize readily. Consequently, another type of experiment was undertaken, one which would allow the isolation of a sufficiently large sample of the product so that its identification could be made unequivocally by means of its mass spectrum.

Large Scale Incubation—To 1.2×10^4 cpm of the [7 α -³H]phenyl analog was added 1.09 mg of unlabeled carrier. The mixture was dissolved in 10 mg of Tween 80 in 5 ml of acetone and the solution transferred to the incubation flask. The acetone was evaporated under a stream of nitrogen and 50 ml of ethylene glycol was added followed by 300 ml of Tris/HCl buffer, pH 7.4. After the suspension was sonified 3 times for 1 min each time, a bovine adrenal mitochondrial acetone powder (500 mg of protein), suspended in 300 ml of the Tris/HCl buffer, was added. This was followed by 100 ml of a TPNH generating mixture (11) and an additional 250 ml of buffer. The mixture, final volume, 1 liter, was incubated at 38° for 1 h in a shaking water bath. The reaction was terminated by the addition of 5 liters of methanol. The alcoholic solution was filtered to remove the precipitated proteins and the volatile solvents of the filtrate evaporated under vacuum. In order to remove the ethylene glycol from the residue, 100 g of celite was added and the mixture packed into a column (2.5-cm diameter) over a layer of 20 g of celite containing 10 g of water. The steroidal products were eluted from the column with 300 ml of isooctane. The hydrocarbon eluate was evaporated to dryness and the residue chromatographed on celite in System C-2 where the nonpolar products were eluted at the end of the 1st holdback volume. The fractions comprising this peak were combined, the solvent evaporated, and the residue chromatographed on a 30-g column in the reverse phase system, C-3, where a single radioactive peak was found in the 4th to 5th holdback volume. The fractions comprising the peak were combined (46,500 cpm), the solvents evaporated to dryness, and the residue rechromatographed on a column (1.2 \times 4 cm) of alumina containing 9% H₂O. The

column was developed first with 50 ml of petroleum ether, then with 50 ml of a mixture of benzene/petroleum ether (1/4) and finally with a mixture of benzene/petroleum ether (1/1) with which the radioactive peak was eluted. The fractions containing the radioactivity (34,100 cpm) were combined and the contents rechromatographed by high pressure liquid chromatography on a μ Bondapak C₁₈ column (12 \times 1/4 inch, Waters Co.) in acetonitrile/H₂O (65/35) at 1 ml/min; 1-ml fractions were collected. The material containing tritium was eluted in Fractions 14 to 17 which were combined. The tritium content was 15,000 cpm, corresponding to a yield of C₁₈ steroid of 9.8 μ g.

Ten per cent of the solution containing the radioactive material was transferred to a conical tube (7 \times 40 mm). The solvent was evaporated and the residue carefully washed to the bottom several times with acetone, evaporating the solvent with a stream of nitrogen each time. The trimethylsilyl ether was prepared by adding 1 μ l of pyridine and 2 μ l of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Pierce Chemical Co.) to the residue. The small conical tube was then placed into a screw-capped vial (15 \times 44 mm) which contained 100 μ l of the silylating reagent in the bottom of the vial. The vial was closed and the mixture heated at 60° for 10 min. Approximately 50% of the reaction mixture was injected into the gas chromatograph section of the gas chromatograph/mass spectrometer. The spiral glass column (72 \times 1/8 inch) was packed with 3% SP-2100 on Supelcoport 100/120 (Supelco) and the chromatogram developed with helium flowing at a rate of 30 ml/min. The injection port was at 260° and the column temperature was programmed to increase from 185° to 250° at 4° per min. The effluent from the column was divided by a stream splitter so that one-third of the fraction went to the flame ionization detector of the gas chromatograph and the remaining two-thirds went to the mass spectrometer through a transfer line kept at 240°. The source of the spectrometer was at 210° where ionization was carried out at 75 eV. The gas chromatograph effluent was scanned automatically at 2 per decade and the data recorded on the computer.

The gas chromatographic analysis (Fig 5) displayed several peaks. The two predominant ones were partially separated; one was eluted at 11.8 min and the other at 12.2 min. The minor peaks were not steroidal (as evidenced by mass spectral analysis) and were not further

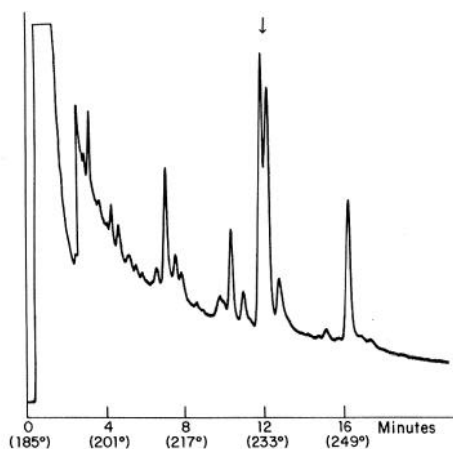


FIG. 5. Gas chromatogram of the trimethylsilyl derivative of the C_{19} steroidal products of the "large scale" incubation. The arrow denotes the two peaks at 11.8 and 12.2 min whose mass spectra are consistent with C_{19} steroids.

examined. The mass spectra obtained by scanning over the entire region of the two peaks eluted at 11.8 and 12.2 min were identical except for minor differences in the relative intensities of the major fragments. A representative mass spectrum of the material in these peaks is shown in Fig. 6a. The trimethylsilyl ether of authentic 1 and 2 both had retention times of 12.2 min in this gas chromatographic system; however 3 emerged at 11.8 min. The mass spectra of the three standards were all similar (Fig. 6, b, c, and d) and were virtually identical with the spectrum obtained from the unknown product(s), (Fig. 6a).

An equimolar mixture of the three synthetic olefins, 1, 2, and 3, was prepared and the mixture subjected to analysis on the gas chromatograph/mass spectrometer. The results were similar to those obtained with the product(s) of the incubation. The gas chromatographic analysis only partially resolved the synthetic mixture. The mass spectrum of the mixture (shown in Fig. 6e) closely resembled that of the biosynthetic product(s). From these results it appears that the biosynthetic product eluted from the gas chromatograph with a retention time of 12.2 min is 1. The identity of the other product with a retention time of 11.8 min was not determined.

In Vitro Incubation of (20S)-20-Hydroxy[21- 14 C]cholesterol—Side chain-labeled, (20S)-20-hydroxy[21- 14 C]cholesterol (3.6×10^6 cpm, 12.7 μ g) was incubated with an acetone powder prepared from bovine adrenal cortex mitochondria (31 mg of protein) and a TPNH generating system for 1 h at 37°. The total volume of the incubation mixture was 1.5 ml. After the incubation, the following carriers were added: 92 mg of 6-methyl-2-heptanone and 24.5 mg of pregnenolone. The mixture was extracted 3 times with 10-ml portions of ether. The ether extract was dried over Na_2SO_4 and evaporated to dryness at low temperature. The oily residue was dissolved in a minimum volume of ether and transferred to a microdistillation unit where the ether was removed under a stream of N_2 . The 6-methylheptanone was then distilled *in vacuo* (less than 1 mm Hg). The distillate was chromatographed on a Silica Gel 60 (Merck) column (35 \times 15 mm). The column was developed first with petroleum ether, then with a mixture of benzene/petroleum ether (1/9) and finally with a mixture of benzene/petroleum ether (3/7) which eluted the ketone. Its semicarbazone was prepared in the usual way and this derivative was chromatographed on the high pressure liquid chromatograph on a Porasil C_{18} column (36 \times 3/8 inch) with acetonitrile/ H_2O (35/65) at a flow rate of 2 ml/min. The C_8 -semicarbazone chromatographed with a retention time of 35 min. The fractions containing this material were combined, the solvents evaporated, and the product crystallized twice (Table II). The crystalline derivative did not reach constant specific activity during the process so that its radiochemical homogeneity was not established. In any event, the amount of radioactivity remaining in the final crystalline product was 2.8 cpm/mg, corresponding to a maximum yield of 372 cpm or 0.01%. Since this amount of radioactivity was insignificant when compared to the amount of pregnenolone formed (see below), no effort was made to purify the sample further.

The steroidal residue left after the distillation of the C_8 carrier was chromatographed on celite in System C-2 where progesterone migrates

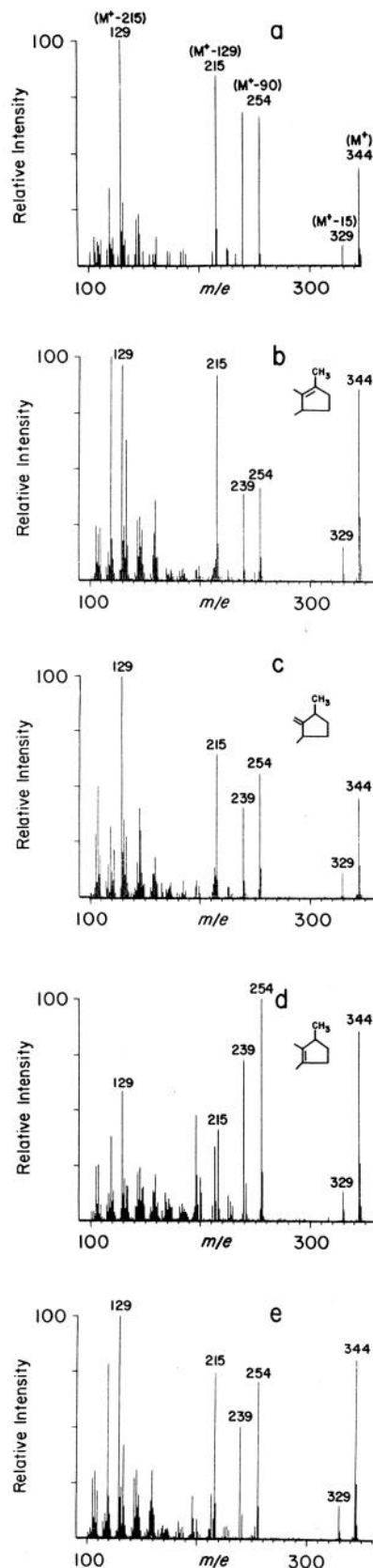


FIG. 6. a, a representative mass spectrum of the gas chromatographic effluents at 11.8 and 12.2 min. b, c, and d, the mass spectra of the gas chromatographic effluents of the trimethylsilyl ethers of 1, 2, and 3, respectively. e, a representative mass spectrum of the gas chromatographic effluent of an equimolar mixture of 1, 2, and 3.

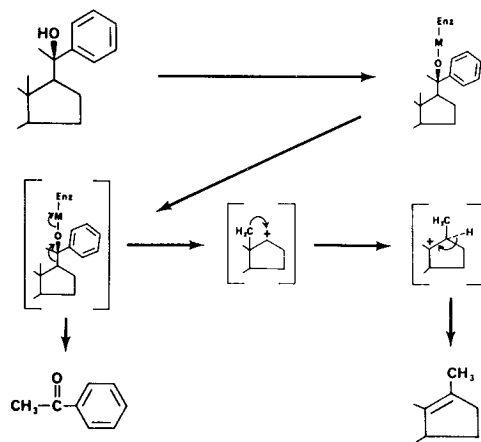


FIG. 7. Proposed mechanism for the biosynthesis of Compound 1 from the phenyl analog. *Enz-M-O-* represents a reactive complex of the oxygenated steroid and the presumed metalloenzyme.

in the 3rd holdback volume and pregnenolone in the 5th and 6th holdback volume. Those fractions containing the carrier pregnenolone were combined, the contents acetylated, and the product chromatographed on celite in System C-4 where pregnenolone acetate is eluted in the 4th and 5th holdback volumes. Those fractions containing this C₂₁ steroidal acetate were combined, the solvents evaporated, and the acetate crystallized to constant specific activity (Table II). The constancy of specific activity was taken as evidence for the biosynthesis of pregnenolone. The specific activity of 37,000 cpm/mg corresponds to a yield of 1.02×10^6 cpm or 29%.

DISCUSSION

Previous observations from this laboratory (1, 8, 13-16) have led us to propose that the steroid hormones are biosynthesized from cholesterol by enzymatic reactions involving transient intermediates bound to multienzyme complexes. This postulate holds that stable, isolable hydroxylated compounds need not be obligatory intermediates in these processes. The results obtained with the phenyl analog of cholesterol, (20*R*)-20-phenyl-5-pregnene-3 β ,20-diol, are illustrative of the kind of evidence that lends support to this thesis. This compound was converted into pregnenolone by adrenal mitochondria in spite of the fact that the carbon atom adjacent to C-20 (which would be C-22 in cholesterol) is a member of the aromatic ring and therefore cannot yield a stable hydroxylated product. The formation of phenol suggests that enzymatic oxygenation of that carbon atom occurs simultaneously with cleavage of the carbon-carbon bond. Cleavage of the C-17,20 bond of the phenyl analog led to two unexpected products, acetophenone and 17-methyl-18-norandrosta-5, 13 (17)-dien-3 β -ol, the latter undoubtedly arising by rearrangement of an intermediate species bearing a positive charge on C-17 (Fig. 7).

Formation of an ionic species probably is facilitated by the neighboring aromatic group but no such facilitation can occur when the substituent on C-20 is the alkyl group, isohexyl, as it is in both cholesterol and (20*S*)-20-hydroxycholesterol. Both of these substances yield, upon incubation with an enzyme preparation from the cortex of the adrenal, pregnenolone and isocaproic acid but neither undergoes scission between C-17 and C-20. The results described in this paper indicate that (20*S*)-20-hydroxycholesterol, like cholesterol, itself, is not oxidized under these *in vitro* conditions to the C₈ product, 6-methyl-2-heptanone.

Reaction of the aromatic substrate with an enzyme (presumably a metalloenzyme) seems to be sufficient to rationalize the formation of acetophenone and the rearranged product, 17-methyl-18-norandrostedienol. Enzymatic oxygenation appears to be unnecessary since transfer of electrons from the substrate to the metal of the metalloenzyme could initiate the process (Fig. 7). Whether the enzyme catalyzing the cleavage of the (C-17,20) bond is the same cytochrome P-450 enzyme as that involved in oxygenation of cholesterol at C-20 is, of course, unknown but this would not be unreasonable since 20-hydroxylation of another aromatic analog of cholesterol, (20*S*)-20-(*p*-tolyl)-5-pregnen-3 β -ol can be effected by a naturally occurring enzyme present in adrenal mitochondria (6). The enzyme involved apparently does not discriminate between the natural substrate and the synthetic aryl derivative. In any event, all the products formed from the phenyl analog can be rationally explained by assuming that the true intermediate is a complex of the steroidal substrate and the metalloenzyme. In one instance, attack by another molecule of activated oxygen leads to the production of pregnenolone and phenol. In the other, a shift of electrons in the complex leads to fission of the C-17,C-20 bond yielding the C₁₈-dienol and acetophenone.

An analysis of the products isolated from the large scale incubation of the phenyl analog with mitochondria from the adrenal cortex revealed that two nonpolar compounds were formed. Analysis by gas chromatography/mass spectrometry indicated that one (retention time 12.2 min) was Compound 1. The material eluted at 11.8 min from the gas chromatogram has not been identified although its chromatographic properties and mass spectrum are similar to those of the C₁₈ steroids (1, 2, and 3) isolated from the Bamford-Stevens elimination reaction. Thus, it is likely that this unidentified substance, is derived from a reactive species left after fragmentation of the C-17,20 bond.

Another aspect of the results reported in this paper is worthy of note. It concerns the efforts made to identify the nonpolar C₁₈-dienol formed from the synthetic phenyl derivative. Following the identification of acetophenone as a by-product of the reaction, it was reasonable to assume that the steroidal segment was the Δ^{16} -olefin, 5,16-androstadien-3 β -ol. Addition of this substance as cold carrier to the trace of radioactive product gave a mixture which after three recrystallizations (from two different solvent systems) and two chromatographic separations (once by partition chromatography and once by thin layer chromatography on silica gel) gave evidence that the radioactive product was identical with the added carrier. Only after further analysis were the two separated from each other. This example shows how difficult it can be to establish the identity of a trace quantity of a radioactive material by reverse isotope dilution techniques. This methodological point is worthy of special attention because it illustrates that great care and persistence are required for reliable identifications. In fact, certainty can never be achieved by means of this convenient technique and, unlike most other analytical procedures, the less care expended in its application, the easier it is to "establish" identity.

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Identification of 17-methyl-18-norandrosta-5,13(17-dien-3beta-ol, the C19 fragment formed by adrenal side chain cleavage of a 20-aryl analog of (20S)-20-hydroxycholesterol.

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