Biosynthesis of Steroid Sulfates by the Boar Testes†

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ABSTRACT: The steroidogenic enzymes present in boar testicular tissue have been shown to use steroid sulfates as substrates. Pregnenolone sulfate, doubly labeled with ^3H in the nucleus and ^{35}S, was incubated with the microsomal fraction of boar testicular tissue and a reduced triphosphopyridine nucleotide generating system and 17-hydroxypregnenolone sulfate as well as dehydroisoandrosterone sulfate were isolated. These products had the same ^3H to ^{35}S ratio as the substrate demonstrating that the conversions had taken place with the sulfate group intact. Thus testicular 17-hydroxylase and C-17,20-desmolase can convert Δ^5-3β-yl sulfates into products which still contain the sulfate group at C-3. An unusual steroid sulfate, doubly labeled 5,16-androstadien-3β-yl sulfate, was also isolated demonstrating that such an olefin can be biosynthesized via steroid sulfate pathways. In a second experiment small amounts of [^3H]-21-hydroxypregnenolone sulfate were isolated from the incubation of [^3H]pregnenolone sulfate with microsomes from boar testes. The isolation of the 21-hydroxylated steroid sulfate is taken as support for the hypothesis that Δ^{16} steroids are biosynthesized from their C_{21} precursors by events that are initiated by oxygenation at C-21.

One of the most abundant secretory products of the adrenals, dehydroisoandrosterone sulfate,[1] can be biosynthesized from cholesterol sulfate by a sequence of reactions, each of which employs sulfated intermediates as substrates (Roberts et al., 1964). Although this C_{19} sulfate can also be formed in the adrenal from its unconjugated C_{19} counterpart by sulfation, an uncertain fraction of the conjugate is derived from sulfate precursors that retain their 3-sulfate groups throughout the entire series of biosynthetic transformations. The work described in this paper was undertaken in order to determine whether the testis also contains steroidogenic enzymes that can use sulfated steroids as precursors. During this study a new transformation was uncovered, pregnenolone sulfate can be converted by incubation with testicular microsomes into 5,16-androstadien-3β-yl sulfate (Figure 1). The precursor, pregnenolone sulfate, was labeled with ^3H and ^{35}S and the isolated unsaturated sulfate contained the two isotopes in the same ratio as that present in the starting material, proving that the sulfate group was retained throughout the reaction sequence (Figure 1). This conjugate had previously been found in human urine and testicular tissue (Ruokonen et al., 1972; Ruokonen, 1973). Although C_{19}-Δ^{16} steroids are pheromones in some mammals, particularly pigs, their physiological roles in humans, where they are found in the urine of both men and women in milligram quantities (Gower, 1972) are unknown.

Two other doubly labeled products were isolated from the incubation mixture. One, dehydroisoandrosterone sulfate, was previously known to be secreted by the testes (Baulieu et al., 1967) but the results presented here provide evidence that the conjugate can be formed, in this tissue, as it is in the adrenals, from sulfate precursors. The other compound was 17-hydroxypregnenolone sulfate, a conjugate previously known to be made in the adrenals from prenenolone sulfate (Calvin and Lieberman, 1964). A sulfokinase that can sulfurylate 17-hydroxypregnenolone is present in testes (Pierrepoint et al., 1966), but this is the first demonstration that the conversion, pregnenolone sulfate → 17-hydroxypregnenolone sulfate, can occur in that organ.

In 1970 it was suggested (Lippman and Lieberman, 1970) that the process which initiates the transformation of pregnenolone into a C_{19}-Δ^{16} olefin is oxygenation at C-21. In order to explore this proposal, an attempt was made in a second incubation experiment to isolate 21-hydroxypregnenolone sulfate which might be formed as a result of that oxygenation. In an experiment using testicular microsomes as the enzyme source and [^3H]pregnenolone sulfate of high specific activity as the substrate, tritiated 21-hydroxypregnenolone sulfate was isolated.

Experimental Section

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 four times reflecting beam condensers. Colorimetric assays were determined with a Zeiss spectrophotometer (PMQ II).

All tracers were purchased from New England Nuclear Corp. Radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer (Model 3375). Labeled free steroids were counted in a toluene-based phosphor scintillation solution. Radioactive steroid sulfates were first dissolved in 1 ml of methanol before the addition of 5 ml of the scintillation mixture. The ^3H and ^{35}S counts were corrected using the method described by Okita et al. (1957).

Partition column chromatography was performed on Celite

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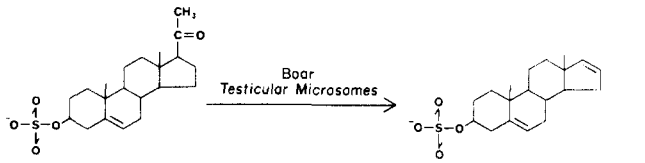
[1] Abbreviations or trivial names used are: dehydroisoandrosterone sulfate, 17-oxo-5-androsten-3β-yl sulfate; dehydroisoandrosterone, 3β-hydroxy-5-androsten-17-one; cholesterol sulfate, 5-cholesten-3β-yl sulfate; pregnenolone sulfate, 20-oxo-5-pregnen-3β-yl sulfate; pregnenolone, 3β-hydroxy-5-pregnen-20-one; 17-hydroxypregnenolone sulfate, the 3-sulfate of 3β,17α-dihydroxy-5-pregnen-20-one; 21-hydroxypregnenolone sulfate, the 3-sulfate of 3β,21-dihydroxy-5-pregnen-20-one; deoxycorticosterone, 21-hydroxy-4-pregnen-3,20-dione; TPNH, reduced triphosphopyridine nucleotide; uv, ultraviolet; ir, infrared.



PREGNENOLONE SULFATE 5,16-ANDROSTADIEN-3β-YL-SULFATE

FIGURE 1.

(Johns Manville No. 545), using previously described techniques (Siiteri, 1963). Stationary phase and Celite were packed in a ratio of 1 ml of stationary phase to 2 g of Celite. The volume of mobile phase retained by the column (holdback volume) was approximately 1.5 times that of the weight of Celite. Adsorption column chromatography was carried out on Woelm neutral alumina (Waters Associates), silica gel B (VWR Scientific), or Florisil, 60–100 mesh (Fisher Scientific). Thin-layer chromatography was carried out on silica gel GF plates (Quantum Industries) and on silica gel GF plates impregnated with 20% $AgNO_3$ prior to use (Lisboa and Palmer, 1967). Reverse-phase, high-pressure liquid chromatography was performed on C_{18} Corasil and C_{18} Porasil B columns (Waters Associates) using a Waters Model ALC 100, high-pressure, liquid chromatograph equipped with refractive index and uv detectors and a Model 660 solvent programmer. All solvents were either of analytical grade or were distilled before use. The chromatographic systems used in this investigation are tabulated in Table I. The methylene blue reagent of Crepy and Rulleau-Meslin (1960) was employed to locate and quantify sulfated steroids.

Preparation of Steroid Sulfates. Sodium [7α-^{3}H]Pregnenolone Sulfate and Sodium Pregnenolone [^{35}S]Sulfate. [7α-^{3}H]Pregnenolone (20Ci/mmol) (purchased from New England Nuclear Corp.) was purified by chromatography on Celite in system C-1 (Table I). Radioactive pregnenolone sulfate was prepared using pyridine–sulfur trioxide, by the procedure previously described (Roberts et al., 1964). The sodium salt of pregnenolone sulfate was extracted from a 15% NaCl solution with two portions of freshly distilled tetrahydrofuran (5 volumes). The organic extract was concentrated to dryness in vacuo and the resulting sodium [7α-^{3}H]pregnenolone sulfate was chromatographed on Celite in system C-2.

Sodium pregnenolone [^{35}S]sulfate was prepared using [^{35}S]chlorosulfonic acid (24.2 mCi/mmol) purchased from New England Nuclear Corp.) by the method previously described (Roberts et al., 1964). The pyridinium salt was converted into the triethylammonium salt by the procedure of Mickan et al. (1969). The triethylammonium salt (Et_3N) of pregnenolone [^{35}S]sulfate was crystallized from acetone and then further purified by chromatography on Celite in system C-3 (Mickan et al., 1969). The triethylammonium salt was converted to the sodium salt by dissolving it in a 15% NaCl solution. The sodium salt was recovered by extraction with 5 volumes of freshly distilled tetrahydrofuran. The solvent was evaporated and the residue dissolved in methanol and stored in a refrigerator. The specific activity of the purified product was 1.89×10^7 cpm/μmol.

Radiochemical homogeneity of each tracer steroid was established by diluting an aliquot of the tracer with an accurately weighed quantity of carrier. The mixture was then recrystallized. Only when the specific activity of the recrystallized product was equal, within experimental error, to that of the original diluted mixture was the tracer considered to be devoid of contaminants and acceptable for further use. Steroid sulfates were crystallized as their triethylammonium salts (Mickan et al., 1969).

TABLE I: Chromatography Systems.

C-1[a]	Isooctane–methanol–water (10:9:1)
C-2	Isooctane–ethyl acetate–*tert*-butyl alcohol–methanol–1% $NaHCO_3$[b] (2.66:4:2:2:3)
C-3	Isooctane–benzene–2-butanone–methanol–0.2 M $(Et_3N)_2SO_4$[c] (2.5:4:2:2:3)
C-4	Isooctane–ethyl acetate–*tert*-butyl alcohol–methanol–1% $NaHCO_3$ (3:3:2:2:3)
C-5	Isooctane–ethyl acetate–*tert*-butyl alcohol–methanol–1% $NaHCO_3$(2:4:2:2:3)
C-6	Isooctane–benzene–2-butanone–methanol–0.2 M $(Et_3N)_2SO_4$ (7.5:3:2:2:3)
C-7	Chloroform–heptane–*tert*-butyl alcohol–1 M NH_4OH[b] (1:2.5:2:2)
C-8	Isooctane–benzene–2-butanone–methanol–0.2 M $(Et_3N)_2SO_4$ (3:3:2:2:3)
C-9	Isooctane–ethyl acetate–*tert*-butyl alcohol–methanol–1% $NaHCO_3$ (2.4:4:2:2:3)
C-10	Isooctane–benzene–2-butanone–methanol–0.2 M $(Et_3N)_2SO_4$ (3.4:4:2:2:3)
C-11	Isooctane–benzene–2-butanone–methanol–0.2 M $(Et_3N)_2SO_4$ (4.5:3:2:2:3)
C-12	Isooctane–benzene–methanol–water (10:1:4:1)
T-1[d]	Methylene chloride–acetone (60:40)
T-2	Ethanol–2-butanone–benzene–water (3:3:3:1)
T-3	Benzene–ethyl acetate (3:1)

[a] C, column-celite partition system: 0.5 ml of stationary phase per g of celite. [b] In water. [c] $(Et_3N)_2SO_4$–triethylammonium sulfate in water. [d] T, thin layer-silica gel GF Visualization: phosphomolybdic acid (10% in EtOH), followed by warming to 100 °C.

Aliquots of sodium [7α-^{3}H]pregnenolone sulfate and sodium pregnenolone [^{35}S]sulfate were combined (^{3}H/^{35}S = 3.9) and rechromatographed on Celite in system C-2. As expected the radioactive peaks of both tracers were found to coincide. This mixture of the two radioactive sulfates was used in the subsequent in vitro experiment.

5,16-Androstadien-3β-yl Sulfate (Shapiro and Heath, 1967). In a round-bottomed flask, 10 g of dehydroisoandrosterone, (35 mmol) and 6.9 g of *p*-toluenesulfonylhydrazine, (37 mmol) were dissolved in 500 ml of absolute ethanol, 4 drops of concentrated H_2SO_4 was added, and the resulting solution was heated under reflux for 14 h. The volume of the reaction mixture was then reduced to about 350 ml. After cooling, the precipitated steroidal tosylhydrazone was collected, recrystallized from ether, and dried overnight under vacuum at 65 °C.

To a suspension of 9.3 g of the steroidal tosylhydrazone in 500 ml of dry ether (freshly distilled from lithium aluminum hydride), 250 ml of *n*-butyllithium (1.6 M in hexane) was added dropwise via an addition funnel over a 60-min period. The reaction mixture was stirred overnight at room temperature and then treated with 500 ml of cold water. The ether layer was separated and the aqueous suspension extracted with two additional volumes of ether. The combined ether extract was concentrated in vacuo to yield an oily residue which was chromatographed on a 200-g silica gel column. The column was prepared in a 1:1 mixture of ether and petroleum ether and the Δ^{16} steroid was eluted with 2 l. of the same solvent mixture. The pooled eluates were evaporated to dryness and the residue purified by rechromatography on a 100-g neutral alumina column containing 6% water. The column was washed first

with petroleum ether, then with petroleum ether–ether (80:20), and finally with petroleum ether–ether (65:35) which eluted the Δ^{16} steroid. The 5,16-androstadien-3β-ol was recrystallized from petroleum ether–ether and then from aqueous MeOH: mp 138–139 °C (lit. 138–139 °C) (Matthews and Hassner, 1972).

The product exhibited a single spot (R_f 0.40, corresponding to an authentic sample) on a silver nitrate impregnated silica gel thin-layer plate with methylene chloride–acetone (60:40) (system T-1) as the eluting solvent. Its infrared spectrum (micro KBr) provided further proof of identity: 3320 (broad, 3β-OH), 3040 (C–H stretch of olefin), no 1700–1750 (carbonyl), 1675 (C=C stretch), 1058 (Δ^5-3β-OH), and 724 cm^{-1} (C–H bend of Δ^{16}), (Roberts et al., 1958; Bellamy, 1958).

5,16-Androstadien-3β-ol (1 g) was sulfurylated with pyridine–sulfur trioxide. The triethylammonium salt of 5,16-androstadien-3β-yl sulfate was prepared as before and was crystallized from methanol–acetone, mp 175–177 °C. The triethylammonium salt migrated as a single spot on a silica gel thin-layer chromatograph in system T-2. The infrared spectrum (micro KBr) showed the following absorption bands: 3030 (C–H stretch of olefin), 2680 (characteristic of Et_3N), 1614 (C=C stretch), 1200–1260 (characteristic of 3-sulfate (Colthup, 1950), and 715 cm^{-1} (C–H bend of Δ^{16}).

21-Hydroxypregnenolone 3β-Sulfate. 21-Acetoxy-3β-hydroxy-5-pregnen-20-one (21-acetoxypregnenolone) was sulfated by treatment with chlorosulfonic acid by the procedure given above. The 21-acetoxy group was removed by saponification: 1.4 g of sodium 21-acetoxypregnenolone 3β-sulfate was dissolved in 600 ml of hot methanol to which was then added slowly 60 ml of a 10% aqueous solution of Na_2CO_3. The reaction mixture was heated at reflux temperature for 1 h and allowed to stand overnight. The pH was lowered to 8 with 6 N HCl and the solvents were evaporated in vacuo until the final volume was 75 ml. The sodium salt was precipitated by the addition of 15 g of NaCl. It was collected and recrystallized from methanol, mp 190–192 °C. A portion of this sample was converted to the triethylammonium salt by the procedure outlined previously and crystallized from acetone, mp 185–187 °C.

The triethylammonium and sodium salts of 21-hydroxypregnenolone 3β-sulfate each migrated as a single spot on silica gel, thin-layer chromatography in system T-2. The infrared spectrum (micro KBr) of the triethylammonium salt showed the following absorption peaks: 3430 (broad, 21-OH), 2680 (characteristic of Et_3N group), 1715 singlet (20-ketone) and 1200–1260 cm^{-1} (3-sulfate).

Preparation of Microsomes from Boar Testis. A boar testis packed in ice at the slaughter house was delivered to the laboratory where it was decapsulated. Fifty grams of tissue was placed in 0.25 M sucrose (5 v/w) and homogenized first by two 30-s bursts in a Waring blender and then by two passes in a motor-driven, Potter-Elvehjem homogenizer. The suspension was centrifuged for 20 min at 9000*g*, yielding a pellet which was resuspended in 2 volumes of 0.25 M sucrose. The suspension was homogenized and recentrifuged for 20 min at 9000*g*. The supernatant thus obtained was centrifuged in a Beckman Spinco (Model L2-75B) ultracentrifuge at 105 000*g* for 90 min. The resulting microsomal pellet was suspended in 10 ml of 0.1 M phosphate buffer, pH 7.4, by sonication at 0 °C for 30 s using a microtip at 60 W. This microsomal suspension was used in subsequent incubation experiments. In control experiments, the suspension was inactivated by boiling aliquots for 15 to 20 min.

General Conditions for Incubation. A typical incubation was performed in the following manner. Equimolar quantities of the substrates dissolved in methanol were added to two tubes (one the control) each containing 0.1 ml of propylene glycol. After mixing, the methanol was evaporated under a stream of nitrogen. To these solutions was added, with thorough mixing, 2.5–4.3 ml of 0.1 M phosphate buffer (pH 7.4 except as noted) so that after the additons of the following solutions a final volume of 5.0 ml was attained. Next, each tube received 0.5 ml of a NADPH-generating system, 12.5 mg (41.2 μmol) of glucose 6-phosphate, 3.75 mg (5 mmol) of NADP, 2.5 units of glucose-6-phosphate dehydrogenase (Sigma Chem Co.), and 0.05 ml of 0.1 M $MgCl_2$ in 0.1 M phosphate buffer, pH 7.4. Finally, the reaction was initiated by the addition of various volumes (0.2 to 2.0 ml) of the microsomal preparation described above to one tube, while the control tube received the same quantity of the previously boiled microsome solution. The mixtures were then shaken at 37 °C for 30 min. The incubations were terminated by the addition of 200 ml of methanol containing appropriate carrier steroids. The aqueous methanolic solutions were filtered to remove the precipitated protein and the precipitates were washed thoroughly with methanol. The aqueous methanolic filtrates were combined and evaporated and the residue was chromatographed as indicated below.

Incubation I. Sodium [7α-^{3}H]pregnenolone sulfate (1.767×10^6 cpm, 91.3 pmol) and sodium pregnenolone [^{35}S]sulfate (4.532×10^5 cpm, 24.0 nmol) ($^3H/^{35}S = 3.9$) were incubated together according to the procedure described above with 0.2 ml of the microsomal preparation, the carriers, sodium 5,16-androstadien-3β-yl sulfate (5 mg, 13.4 μmol), sodium pregnenolone sulfate (5 mg, 11.9 μmol), sodium 17-hydroxypregnenolone 3-sulfate (5 mg, 11.5 μmol), and sodium dehydroisoandrosterone sulfate (5 mg, 12.8 μmol), were added to each incubation mixture after termination of the reactions. The residue from the methanol extract was chromatographed on Celite in system C-4. Three radioactive zones were eluted with 22 holdback volumes of mobile phase. Zone 1, eluted in the first holdback volume, contained a small amount of unconjugated ^{3}H-labeled steroid (ca. 1% of the total tritium eluted from the column) which probably was liberated from the sulfates during the incubation or subsequent work-up. Zone 2 (^3H = 23 700 cpm and ^{35}S = 5700 cpm), was eluted in the 7–8th holdback volumes where the added carrier, 5,16-androstadien-3β-yl sulfate, was found. Further proof of the identity of this radioactive product is given below. Zone 3 ($^3H = 7.77 \times 10^5$ cpm and $^{35}S = 1.74 \times 10^5$ cpm), eluted in the 15–19th holdback volumes, was found to contain the substrate, sodium pregnenolone sulfate. The appropriate fractions of zone 3 were pooled and evaporated to dryness and the residue was rechromatographed on Celite in system C-2. The radioactivity peak was coincident with the carrier sulfate, as detected by the methylene blue assay.

The column wash ($^3H = 4.49 \times 10^5$ cpm and $^{35}S = 1.25 \times 10^5$ cpm) of the first Celite column (system C-4) contained the 17-hydroxypregnenolone 3-sulfate and dehydroisoandrosterone sulfate carriers. The material present was rechromatographed on Celite in system C-5. Four radioactive peaks were eluted with nine holdback volumes of mobile phase. Peak 1 ($^3H = 3.58 \times 10^5$ cpm and $^{35}S = 9.46 \times 10^4$ cpm) was eluted in the second holdback volume and contained both the 17-hydroxypregnenolone 3-sulfate and dehydroisoandrosterone sulfate carriers. Further analysis of this peak is described below. The three other radioactive peaks were not further investigated.

Characterization of Sodium 5,16-Androstadien[^3H]-3β-yl [^{35}S]Sulfate. Radioactive zone 2 from the Celite chromato-

TABLE II: Crystallization Data.

Sample	Fraction[a]	Solvent[b]	Amount			Sp Act.[c]		
			Mg	^{3}H cpm	^{35}S cpm	^{3}H (cpm/mg)	^{35}S (cpm/mg)	^{3}H/^{35}S[d]
Incubation I								
5,16-Androstadien-3β-yl sulfate	X-1	A-P	0.6600	201	56	305 (138)	85	3.6
	X-2	A-P	0.7870	238	66	302 (137)	84	3.6
	ML-2		0.8840	375	92	424 (192)	104	4.1
	X-3	A-P	1.1640	345	90	296 (134)	77	3.8
	ML-3		0.9480	308	85	325 (147)	90	3.6
	X-4	A-P	1.0280	307	83	299 (135)	81	3.7
	ML-4		1.0375	314	89	303 (138)	86	3.6
5,16-Androstadien-3β-ol	X-1	An-W	0.7952	355		446 (122)		
17-Hydroxypregnenolone 3-sulfate	X-1	M	1.0312	1476	378	1431	367	3.9
	ML-1		1.0539	2091	531	1984	504	3.9
	X-2	M	1.0636	1512	404	1422	380	3.7
	ML-2		1.0590	1614	414	1524	391	3.9
	X-3	M	1.3650	1936	517	1418	379	3.7
	ML-3		1.3039	1905	514	1461	394	3.7
	X-4	M	0.9622	1358	357	1411	371	3.8
	ML-4		0.9821	1448	381	1474	388	3.8
	X-5	M	0.9142	1309	341	1432	373	3.8
	ML-5		0.7365	1062	278	1442	377	3.8
17β-Hydroxy-5-androsten-3β-yl sulfate	X-1	M-A	1.0330	463	126	448	122	3.7
	ML-1		1.0835	308	81	285	75	3.8
	X-2	M-A	0.9730	430	113	442	116	3.8
	ML-2		1.0780	449	118	416	109	3.8
	X-3	M-A	1.0130	450	122	444	120	3.7
	ML-3		1.0150	449	114	442	112	3.9
	X-4	M-A	1.0140	461	124	455	122	3.7
	ML-4		1.0850	479	120	441	111	3.9
Incubation II								
3β,21-Diacetoxy-5-pregnen-20-one	X-1	B-P	2.011	79		39		
	ML-1		2.035	101		50		
	X-2	B-P	2.440	98		40		
	ML-2		2.161	92		43		
	X-3	B-P	2.417	90		37		
	ML-3		2.391	106		44		
	X-4	B-P	2.001	77		38		
	ML-4		1.980	80		40		

[a] X-*n*, crystalline product from the *n*th crystallization; ML-*n*, residue left in mother liquor from the *n*th crystallization. [b] A, acetone; P, petroleum ether (30–60 °C); An, acetonitrile; W, water; M, methanol; B, benzene. [c] Data in the brackets are in cpm/μmol. [d] The ^{3}H/^{35}S ratio of the substrate was 3.8.

gram (system C-4) contained the carrier, 5,16-androstadien-3β-yl sulfate. It was evaporated to dryness and the residue was converted into its triethylammonium salt. This was chromatographed on Celite in system C-6 where radioactivity and steroid sulfate carrier were eluted together in the 3–4th hold-back volumes. The appropriate fractions were pooled and evaporated and an aliquot was taken for quantification by the method of Crepy and Rulleau-Meslin (1960). It was determined that 58% of the carrier was recovered and an additional quantity of the carrier, the triethylammonium salt of 5,16-androstadien-3β-yl sulfate, was added to bring the total weight of the carrier to 28.5 mg. The sample was then recrystallized from acetone–petroleum ether until constant specific activities with respect to both ^{3}H and ^{35}S (Table II) were obtained. This evidence was taken as proof that the isotopically labeled product was identical with the added carrier. The ^{3}H/^{35}S ratio of the C_{19}-Δ^{16} steroid sulfate produced in the incubation was the same as that of the substrate, doubly labeled pregnenolone sulfate, thus demonstrating that the conversion had occurred *via intermediates that had retained* the sulfate group throughout the process.

Additional proof of the identity of the radioactive product was obtained after solvolysis and chromatographic purification of the unconjugated parent steroid. The sulfate and the residues from its mother liquors were combined and converted into the pyridinium salt by dissolving the sample in 50 ml of a 0.6 *M* solution of pyridinium sulfate. The salt was extracted into chloroform after which the organic solvent was evaporated. The resulting pyridinium salt of the steroid sulfate was solvolyzed by treating it with 100 ml of anhydrous tetrahydrofuran overnight at room temperature. After the solvent was removed the unconjugated steroid was purified by preparative thin-layer chromatography on silver nitrate impregnated silica gel plates in system T-1. In this system the Δ^{16} olefin has a R_f 0.40. The steroid was extracted from the silica gel with methylene chloride and then purified again by reverse-phase, high-pressure, liquid chromatography on a C_{18} Corasil column using acetonitrile–H_2O (70:30) as eluent. With a flow rate of 0.5 ml/min, the Δ^{16} steroid was eluted from the column after 10.5 min. The purified compound was then crystallized from acetonitrile and H_2O. As shown in Table II the specific activity with regard to tritium was similar throughout all of these manipulations. The yield of the Δ^{16}-steroid sulfate from pregnenolone sulfate was 0.83%.

Characterization of Sodium [7α-^3H]-17-Hydroxypregnenolone 3-[^{35}S]Sulfate: Peak 1 (from partition chromatogram on Celite using system C-5) containing the 17-hydroxypregnenolone 3-sulfate and dehydroisoandrosterone sulfate carriers was converted into ammonium salts which were rechromatographed on Celite in system C-7 where two radioactive peaks were eluted. The first peak ($^3H = 4.40 \times 10^4$ cpm and $^{35}S = 1.15 \times 10^4$ cpm), eluted in the 3rd holdback volume, coincided with dehydroisoandrosterone sulfate carrier, and its characterization will be described below. The second peak ($^3H = 2.80 \times 10^5$ cpm and $^{35}S = 7.15 \times 10^4$ cpm), eluted in the fifth holdback volume, coincided with 17-hydroxypregnenolone 3-sulfate carrier. The appropriate fractions of this peak were combined and evaporated to dryness and the residue was treated with a mixture of pyridine in acetic anhydride (2:1) for 1.5 h at 50 °C. This acetylation step was interposed to derivitize any contaminating C_{19} 17-hydroxysteroids, which as acetates are easily separable from 17-hydroxypregnenolone sulfate (which is not acetylated by this procedure). After evaporation of the unused reagents the residue was dissolved in a 0.6 M triethylammonium sulfate solution from which the Et_3N salts were extracted by chloroform. The organic soluble material was rechromatographed on Celite in system C-3. The radioactivity and the Et_3N salt of pregnenolone 3-sulfate carrier were eluted together in the 6–9th holdback volumes. The sample was converted to its ammonium salt with ethanolic NH_4OH. It was determined that 32% of the carrier was recovered and additional carrier was added to bring the total weight of the carrier to 51.6 mg. The sample was then recrystallized from methanol to a constant specific activity with respect to both ^3H and ^{35}S as shown in Table II. The $^3H/^{35}S$ ratio of this product was identical with that of the substrate. The yield of 17-hydroxypregnenolone 3-sulfate was 12.8%.

Characterization of Sodium [7α-^3H]Dehydroisoandrosterone [^{35}S]Sulfate. The dehydroisoandrosterone sulfate peak (peak 1, system C-7) was converted into its Et_3N salt which was rechromatographed on Celite in system C-8. The radioactivity and carrier were isolated together in the 5–6th holdback volumes. To aid in characterization, androstanediol 3-monosulfate was prepared by reduction with sodium borohydride. The residue from the pooled fractions was dissolved in 10 ml of cold methanol to which was added 10 mg of sodium borohydride. After standing at 0 °C for 2 h, 50 ml of a hot 20% NaCl solution was added. The solution was extracted three times with two volumes of freshly distilled tetrahydrofuran. The organic extracts were filtered through phase-separating paper (Whatman No. 1 PS), and then evaporated to dryness. The resulting product, androstenediol 3-sulfate, was rechromatographed on Celite in system C-7. The androstenediol 3-sulfate carrier and radioactivity were eluted together in the 6–7th holdback volumes. The sample was converted into its triethylammonium salt and quantified. It was determined that 43% of the carrier was recovered and additional carrier was added to bring its total weight up to 32.6 mg. The sample was then recrystallized from methanol–acetone to a constant specific activity with respect to ^3H and ^{35}S as shown in Table II. The $^3H/^{35}S$ ratio of the product was identical with that of the substrate. The yield of dehydroisoandrosterone sulfate was 1.96%.

A control experiment was carried out using incubation conditions identical with those of incubation I except that the microsomal preparation had been previously heated in a steam bath for 30 min. Except for pregnenolone sulfate, the substrate, all added carriers were reisolated devoid of radioactivity.

Incubation II. Sodium [7α-^3H]pregnenolone sulfate (25×10^6 cpm, 1.16 nmol) was incubated with 2.0 ml of microsomal enzyme preparation according to the procedure described above with the exception that the pH of the medium was 7.9 instead of 7.4 as it had been in incubation I. Sodium 5,16-androstadien-3β-yl sulfate (5 mg, 13.4 μmol), sodium pregnenolone sulfate (5 mg, 11.9 μmol), sodium 17-hydroxypregnenolone 3-sulfate (5 mg, 11.5 μmol), sodium dehydroisoandrosterone sulfate (5 mg, 12.8 μmol), and sodium 21-hydroxypregnenolone 3-sulfate (8 mg, 18.4 μmol) all dissolved in 100 ml of methanol, were added as carriers. After removal of the precipitated proteins by filtration and evaporation of the volatile solvents, the residue was chromatographed on Celite in system C-4. Two radioactive zones were eluted: zone 1 (1st holdback volume, $^3H = 3.21 \times 10^5$ cpm) contained a small amount of ^3H-labeled unconjugated steroid and zone 2 (6–8th holdback volumes, $^3H = 2.31 \times 10^5$ cpm) contained ^3H-labeled 5,16-androstadien-3β-yl sulfate. Further analysis of zone 2 indicated that this C_{19}-Δ^{16} steroid sulfate was formed in 0.69% yield.

The material in the methanol wash of the above Celite column was rechromatographed on Celite in system C-9 by means of which the following compounds were separated: pregnenolone sulfate (2–4th holdback volumes); 17-hydroxypregnenolone 3-sulfate and dehydroisoandrosterone sulfate (5th holdback volume); and 21-hydroxypregnenolone 3-sulfate (6–8th holdback volumes). The compounds were purified to constant specific activity as described above for the products formed in incubation I. The percentage yield of 17-hydroxypregnenolone sulfate was 7.4%; that of dehydroisoandrosterone sulfate was 1.2%.

The fractions containing 21-hydroxypregnenolone 3-sulfate were converted to the ammonium salt and chromatographed on Celite in system C-7. Ammonium 21-hydroxypregnenolone 3-sulfate isolated from 4–5th holdback volumes was acetylated (in pyridine–acetic anhydride (2:1), at 50 °C for 1.5 h), and then converted into its triethylammonium salt. This was chromatographed on Celite in system C-10. The carrier was found (by the methylene blue test) to be in the 3rd holdback volume. This material was rechromatographed on Celite in system C-11 where triethylammonium 21-acetoxypregnenolone 3-sulfate was eluted in the 6–7th holdback volumes. Additional purification of the radioactive compound was obtained after solvolysis and chromatographic separation of the unconjugated steroid. The 21-acetoxypregnenolone 3-sulfate residue was converted into its pyridinium salt as before and this compound was dissolved in 100 ml of anhydrous tetrahydrofuran and kept overnight at room temperature. The resulting free steroid 21-acetoxypregnenolone was chromatographed on Celite in system C-12 where the carrier and radioactivity were eluted in the 6–8th holdback volumes. The radioactive fraction was then chromatographed by reverse-phase, high-pressure, liquid chromatography on a C_{18} Porasil B column. The eluting solvent consisted of a gradient of acetonitrile in H_2O where the organic component increased in a linear fraction from 45 to 80% in 30 min using a flow rate of 2.6 ml/min. The steroid was eluted from the column after 26–28 min when the eluent was 75% acetonitrile and 25% water. This 21-monoacetate was acetylated in pyridine–acetic anhydride (2:1) at 50 °C for 1.5 h. After removal of the unused reagents under a stream of nitrogen, the 3,21-diacetate was chromatographed on a 20-g Florisil column prepared in a mixture of heptane and benzene (3:1). By using increasing amounts of benzene followed by increasing amounts of ethyl acetate in benzene, the diacetate was eluted by a 2% ethyl acetate solution in benzene. A thin-layer chromatogram on silica gel (system T-4) of an

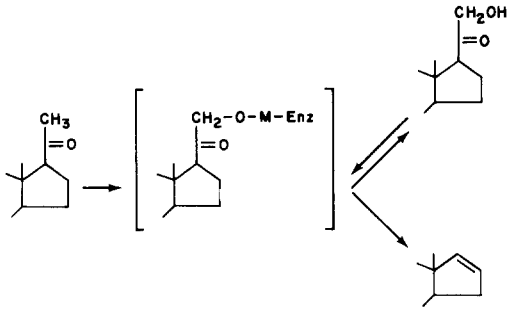


FIGURE 2: Proposed common biosynthetic intermediate for the formation of Δ^{16}-C_{19} steroids and 21-hydroxy-C-21 steroids. The figure in the bracket represents a reactive complex of an oxygenated steroidal species and a metalloenzyme. (M-Enz).

aliquot of the eluted material indicated a single spot (R_f 0.58) corresponding to an authentic sample. An aliquot of the pooled fractions from the Florisil column was taken for quantification of the carrier by the cupric acetate–sulfuric acid assay of Roberts et al. (1969). It was determined that 12% of the carrier was recovered and after additional carrier was added to bring the total weight of the sample to 40 mg, the diacetate, 3β,21-diacetoxy-5-pregnen-20-one, was recrystallized from benzene–petroleum ether to a constant specific activity (Table II) indicating that the labeled product was identical with the unlabeled carrier. The yield of 21-hydroxypregnenolone 3-sulfate was calculated to be approximately 0.05%.

As before, a control incubation using boiled tissue led to the reisolation of the carrier steroids devoid of radioactivity.

Discussion

The results obtained in this study contribute three new facts to our knowledge of the biosynthesis of steroids by the mammalian testes. To begin, these experiments have shown that the sulfate of a C_{19}-Δ^{16} steroid, 5,16-androstadien-3β-yl sulfate, can be formed from pregnenolone sulfate by a pathway involving intact steroidal sulfates. The 3-sulfate group of the intermediates involved in the enzymatic processes by which the olefin is formed remains intact since the product possessed a $^3H/^{35}S$ ratio identical with that of the doubly labeled precursor.

Secondly, 21-hydroxypregnenolone sulfate was shown to be produced from pregnenolone sulfate by incubation with microsomes from testes. That a 21-hydroxylase is present in testes had already been shown by Dominguez et al. (1960) who isolated deoxycorticosterone following incubation of progesterone with testicular microsomes. Our finding was interesting for several reasons. It demonstrated that the testicular 21-hydroxylase could use a sulfated substrate, the conjugate of a 3β-hydroxy-Δ^5 compound, whereas the adrenal hydroxylase employs Δ^4-3-ketones but not 3β-hydroxy-Δ^5 steroids as reactants (Cooper et al., 1968). In addition, the isolation of 21-hydroxypregnenolone sulfate from an incubation mixture which converted the precursor, pregnenolone sulfate, into the olefin, 5,16-androstadien-3β-yl sulfate, provides a clue as to the mechanism by which the $C_{21}O_2$ precursor is converted into the C_{19} olefin. In 1970 Lippman and Lieberman proposed that the proximal precursor of the olefin was a transient species produced by an oxygenation process occurring at C-21 of pregnenolone. Supplemented by other kinds of evidence (Luttrell et al., 1972; Hochberg et al., 1972, 1974, 1975), the hypothesis was broadened to hold that the true intermediates by which each steroid hormone is biosynthesized are short-lived, reactive species in which the steroidal intermediates are complexed with the components, enzymes, cofactors, oxygen, etc., necessary for the formation of the hormone. The suggestion was made that these various components are arranged as structured multienzyme complexes and that the biosynthetic sequence occurs as a series of concerted reactions rather than as stepwise conversions. In this view some of the hydroxylated products which have been isolated from in vitro incubations such as those described in this paper or from urine are not the true intermediates. Rather they are formed, perhaps inadvertently, by hydrolysis of the true intermediates, the steroid–metalloenzyme–oxygen complexes. The isolation of hydroxylated products, of course, provides inferences about the structures of the intermediary complexes which presently are difficult to represent by traditional formulas. The scheme shown in Figure 2 indicates how this concept applies to the formation of both the Δ^{16}-olefin sulfate and 21-hydroxypregnenolone sulfate from a common precursor intermediate derived from pregnenolone sulfate. We have already shown (Lippman and Lieberman, 1970) that a 21-hydroxy-20-ketone, deoxycorticosterone, can serve as an in vitro precursor of the corresponding Δ^{16} steroid, but, as is evident from Figure 2, a stable isolable compound having this structural feature need not be an obligatory intermediate in the conversion. Nevertheless the isolation of 21-hydroxypregnenolone sulfate from the incubation mixture along with the olefin lends some credibility to our hypothesis. Activation and removal of a hydrogen atom or a proton from C-16 of the complex (in which the steroid is bound through oxygen at C-21 to the enzyme complex), perhaps by a nucleophilic group present on the not-too distant enzyme itself, could serve to initiate the cleavage of the C-17–C-20 bond and the resulting formation of the double bond between C-16 and C-17.

Finally these experiments have shown that testicular microsomes can transform a sulfated precursor, pregnenolone sulfate, into other sulfated products, i.e., 17-hydroxypregnenolone sulfate and dehydroisoandrosterone sulfate. In this respect the microsomes of the testes are like those of the adrenals which long have been known to carry out similar conversions. Like the adrenals, the testes are also known (Baulieu et al., 1967) to secrete dehydroisoandrosterone sulfate into the venous effluent, although the function this product serves is still obscure.

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Role of Cyclic Electron Transport in Photosynthesis as Measured by the Photoinduced Turnover of P_{700} in Vivo†

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ABSTRACT: The light-induced turnover of P_{700} was measured spectrophotometrically in a wide variety of algae and some photosynthetic mutants. Analysis of the postillumination recovery of P_{700}^+ revealed that the apparent first-order rate constant for reduction via the cyclic pathway was much lower than that via the noncyclic pathway. After activation of photosystems 1 and 2 the half-time for reduction of P_{700}^+ was 5–20 ms, whereas after activation of primarily photosystem 1 a longer half-time of ca. 150 ms was observed. The extent of the photooxidation of P_{700} was the same in both regimes of illumination. The longer half-time was also noted after inhibition of photosystem 2 by 3-(3,4-dichlorophenyl)-1,1-dimethylurea or mild heat shock and in mutant algae known to lack a functional photosystem 2. No signal was observed in mutants lacking P_{700} itself but those strains lacking either plastocyanin or cytochrome *f* were capable of a very slow turnover (reduction $t_{1/2} > 500$ ms at room temperature). This very slow turnover was not affected by carbonyl cyanide *m*-chlorophenylhydrazone or the plastoquinone antagonist, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, indicating that the pathway for reduction of P_{700}^+ in these mutants is not energy linked and does not utilize the intersystem electron transport chain. The slow, 150 ms, reduction of P_{700}^+ due to cyclic flow was not observed when cells were engaged in photosynthesis at high-light intensities. The data are interpreted as evidence for the involvement of the total functional pool of P_{700} in both electron transport pathways, and we suggest that cyclic electron transport does not contribute to photosynthesis in oxygen-evolving autotrophs.

The assimilation of carbon dioxide by oxygen-evolving photoautotrophs is driven by ATP and $NADPH_2$ that are generated as a consequence of photochemically-induced electron transport reactions by thylakoids. Two major pathways have been demonstrated in vitro: noncyclic photophosphorylation, which produces ATP and $NADPH_2$ via the interaction of two photochemical reactions, and cyclic photophosphorylation, which only produces ATP, in a reaction driven by the long wavelength PS1.[1]

Both pathways have been shown to operate in vivo. However, the experimental demonstration of the cyclic pathway in intact cells requires physiological conditions that may have limited relevance to those that exist during the photosynthesis of carbon dioxide. The possible contribution of the cyclic pathway to photosynthesis itself is uncertain, although it has been demonstrated that this pathway can be utilized for a wide variety of energy-linked functions (Simonis and Urbach, 1973).

A previous report from this laboratory described a kinetic analysis of the photo-induced turnover of cytochrome *f* in *Porphyridium cruentum* during cyclic and noncyclic electron transport (Biggins, 1973). It was concluded from direct spectrophotometric measurements on intact cells that cyclic electron transport does not contribute to the photoassimilation of carbon dioxide. However, the extensive overlap of the absorption bands in the cytochrome Soret and α regions precluded a similar spectrophotometric analysis in other species.

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[1] Abbreviations used are: PS1, photosystem 1; PS2, photosystem 2; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ATP, adenosine 5′-triphosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.