Evidence that Steroid Sulfates Serve as Biosynthetic Intermediates.* IV. Conversion of Cholesterol Sulfate *in vivo* to Urinary C_{19} and C_{21} Steroidal Sulfates†

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Sodium cholesterol-7α-[^{3}H] [^{35}S]sulfate was injected via catheterization of the splenic artery, the vessel supplying 90% of the blood to a massive adrenocortical carcinoma, in a female patient. After extraction of the first 24-hour urine collection with ether-ethanol (3:1), 2.3% of the injected radioactivity was recovered. Of this extracted radioactivity, approximately 20% was recovered as dehydroisoandrosterone sulfate, the major metabolite, bearing ^{3}H and ^{35}S in the same ratio as the injected precursor. In addition, three more polar metabolites have been identified as Δ^5-androstene-3β,17β-diol-3-monosulfate (ammonium salt, mp 210–213°), Δ^5-pregnene-3β,17α,20α-triol-3-monosulfate (ammonium salt, mp 179–181°), and 16α-hydroxydehydroisoandrosterone-3-monosulfate. Identification in each case was achieved by infrared analysis while radiochemical homogeneity was established by crystallization of both the parent compounds and after the formation of appropriate derivatives. Each contained ^{3}H and ^{35}S in essentially the same ratio as that present in the injected material. The isolated dehydroisoandrosterone sulfate, Δ^5-androstenediol sulfate, and pregnenetriol sulfate had the same specific activities with respect to both isotopes. This indicates that the isolated products have a common precursor which is sulfated and that the contribution from 3β-hydroxy-Δ^5-precursors to these compounds must have been small. These findings add further support to the concept that sulfated intermediates may play an active role in the biosynthesis of the steroid hormones.

Since the finding that dehydroisoandrosterone sulfate[1] is a major secretory product of the human adrenal gland (Baulieu, 1962; VandeWiele *et al.*, 1963) attention has been focused on its mode of biosynthesis. Two possible pathways appear reasonable. In one dehydroisoandrosterone, after its synthesis in the adrenal gland, is sulfated in that gland prior to its secretion. That the adrenal gland is indeed capable of sulfating dehydroisoandrosterone has been shown by several groups (Wallace and Lieberman, 1963; Cohn *et al.*, 1962; Migeon, 1963) in *in vitro* experiments using homogenates of human adrenal tumors. On the other hand, the observation that conjugation does not necessarily destine a steroid for excretion (Roberts *et al.*, 1961) led to the consideration of a second pathway in which sulfates *per se* may be involved as intermediates in the biosynthesis of dehydroisoandrosterone sulfate. Two recent findings have supported this idea. Calvin *et al.* (1963) provided evidence that dehydroisoandrosterone sulfate can be synthesized from pregnenolone sulfate *in vivo* by a pathway involving intact steroid sulfates. Subsequently it was also shown that a homogenate of human hyperplastic adrenal tissue has the capacity to hydroxylate pregnenolone sulfate at C-17 with the resulting formation of 17α-hydroxypregnenolone-3-sulfate (Calvin and Lieberman, 1964).

To add further support to these findings and to establish whether pathways involving sulfates were also employed in the biosynthesis of pregnenolone sulfate, sodium cholesterol-7α-[^{3}H] [^{35}S]sulfate has been examined in order to determine whether it too could serve as a sulfated intermediate. After the injection of cholesterol sulfate (^{3}H/^{35}S = 5.7) into the blood supplying a massive adrenocortical carcinoma in a female patient, four radioactive sulfated metabolites, each bearing essentially the same isotopic ratio as the injected material, have been isolated from the first 24-hour urine collection. The metabolites identified were: dehydroisoandrosterone sulfate, Δ^5-androstene-3β,17β-diol-3-monosulfate, 16α-hydroxydehydroisoandrosterone-3-monosulfate, and Δ^5-pregnene-3β,17α,20α-triol-3-monosulfate.

EXPERIMENTAL

Melting points were determined on a Kofler block and are corrected. Infrared spectra were obtained using a Perkin-Elmer Model 221 spectrometer. All solvents were of analytical grade.

Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer (Model 314-DC) at high voltage tap 7 (1120 v), using discriminator settings of 5 v to 50 v and 100 v to infinity for the determination of ^{3}H and ^{35}S, using the method of Okita

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[1] The following trivial names have been used in the text: dehydroisoandrosterone = 3β-hydroxy-5-androsten-17-one; dehydroisoandrosterone sulfate = 3β-hydroxy-5-androsten-17-one-3-sulfate (17-oxo-5-androsten-3β-yl sulfate); pregnenolone = 3β-hydroxy-5-pregnen-20-one; pregnenolone sulfate = 3β-hydroxy-5-pregnen-20-one-3-sulfate (20-oxo-5-pregnen-3β-yl sulfate); 17α-hydroxypregnenolone = 3β,17α-dihydroxy-5-pregnen-20-one; 17α-hydroxypregnenolone sulfate = 3β,17α-dihydroxy-5-pregnen-20-one-3-sulfate; 16α-hydroxydehydroisoandrosterone = 3β,16α-dihydroxy-5-androsten-17-one; 16α-hydroxydehydroisoandrosterone sulfate = 3β,16α-dihydroxy-5-androsten-17-one-3-sulfate; androstenediol sulfate = 5-androstene-3β,17β-diol-3-sulfate.

et al. (1957). Owing to the insolubility of the conjugates in the usual toluene phosphor solution, samples of conjugates to be counted were dissolved in 1 cc of methanol in glass vials prior to the addition of 5 cc of the phosphor solution (0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazolyl)benzene) (Packard Chemicals). Appropriate corrections were made for interference of one isotope with the measurement of the other, for the decay in the samples containing ^{35}S, and for quenching.

Column partition chromatography on celite (Johns Manville No. 545) was used throughout. Preparation of the columns has been described previously (Siiteri, 1963). Stationary phase and celite were packed in a ratio of 1 ml of stationary phase to 2 g of celite, and the volume of mobile phase retained by the column (holdback volume) was approximately 1.5 times that of the weight of celite. The chromatographic systems used in this investigation are tabulated in Table I and are referred to in the text by code letter. The eluants moved through the columns at a rate of approximately 1 holdback volume per hour. Each chromatogram was followed by radioactive assay of aliquots of the fractions. In addition, the methylene blue reagent of Vlitos (1953), as modified by Roy (1956) and Crepy and Rulleau-Meslin (1960), was employed to locate sulfated metabolites.

TABLE I
SYSTEMS USED FOR PARTITION CHROMATOGRAPHY ON CELITE

System	Components
A	Methylcyclohexane 3, ethyl acetate 2, methanol 3, 1 M NH_4OH 3
B	Heptane 4, 1-butanol 1, methanol 2, 0.3 M pyridinium sulfate 2, pyridine 0.1
C	Isooctane 2, *t*-butyl alcohol 5, 1 M NH_4OH 5
D	Isooctane 4, chloroform 2, 1-butanol 0.7, methanol 2, 0.3 M pyridinium sulfate 2, pyridine 0.1
E	Isooctane 2, ethyl acetate 4, 1-butanol 1, methanol 2, 1 M NH_4OH 3
F	Isooctane 2.5, ethyl acetate 4, 1-butanol 0.8, methanol 2, 1 M NH_4OH 3
G	Isooctane 2, ethyl acetate 4, 1-butanol 1.5, methanol 2, 1 M NH_4OH 3
H	Isooctane 4, chloroform 2, 1-butanol 1, methanol 2, 0.3 M pyridinium sulfate 3, pyridine 0.1
J	Ethyl acetate 4, 1-butanol 1, methanol 1, 1 M NH_4OH 4

Sodium Cholesterol-7α-[^{3}H] Sulfate and Sodium Cholesterol [^{35}S]Sulfate.—To 1.7 mg of cholesterol-7α-[^{3}H] (1 mc) purchased from the New England Nuclear Corp. was added 5 mg of carrier cholesterol (purified via the dibromide). Crystallization from acetone yielded a sample of cholesterol containing 144×10^6 cpm. This material was dissolved in 0.5 ml of dry pyridine and treated with a solution of 0.05 ml of chlorosulfonic acid in 0.5 ml of dry pyridine. The mixture was heated on a steam bath for 10 minutes and then poured into 20 ml of $CHCl_3$. After the solution was washed with water, the solvent was removed and the crude pyridinium cholesterol sulfate was chromatographed on 30 g of celite using system B. The peak of radioactivity appeared in the 4th–5th holdback volume. The peak fractions, containing 103×10^6 cpm, were combined and the pyridinium salt therein was converted to the sodium salt by treatment with a sodium chloride solution (2 g in 10 ml of water). The sodium salt was recovered by extraction into 1-butanol. The organic extract was washed with water and then evaporated to dryness, yielding 1 mg sodium cholesterol-7α-[^{3}H] sulfate containing 98.6×10^6 cpm of tritium.

Sodium cholesterol [^{35}S]sulfate was prepared in the following manner: 53 mg (5 mc) of [^{35}S]chlorosulfonic acid (purchased from New England Nuclear Corp.) was added to 0.2 ml of dry pyridine and aliquots of this complex were used to prepare various labeled sulfates. A 0.07-ml aliquot of this complex was added to 50 mg of cholesterol (purified via the dibromide) dissolved in 0.2 ml of pyridine. After the reaction mixture was heated on a steam bath for 10 minutes, 10 volumes of 7 M NH_4OH was added and the aqueous solution was extracted twice with 1-butanol. The butanol extracts were washed with water and evaporated to dryness, and the residue was chromatographed on 70 g of celite using system A. This system was found to have a low capacity; the isolated ammonium cholesterol [^{35}S]sulfate (79×10^6 cpm, specific activity 2.4×10^6 cpm/mg) appeared in a broad area between holdback volumes 2 and 6.

A portion of this material was converted to the pyridinium salt by extraction into chloroform from 0.3 M pyridinium sulfate solution (McKenna and Norymberski, 1960). This salt was chromatographed on 50 g of celite using system B. The eluted radioactive pyridinium cholesterol sulfate, which was eluted in holdback volumes 3.5–5, was converted to the sodium salt using the method described previously. The purified product contained 14.5×10^6 cpm of ^{35}S (specific activity 1.9×10^6 cpm/mg).

Aliquots of the sodium cholesterol-7α-[^{3}H] sulfate and sodium cholesterol [^{35}S]sulfate were combined and rechromatographed on system B. The radioactive peaks of both isotopes were found to coincide exactly, establishing the radiochemical homogeneity of the samples. This mixture of the two radioactive sulfates was used in the subsequent *in vivo* experiment.

Injection of Sodium Cholesterol-7α-[^{3}H] [^{35}S]Sulfate.—Doubly labeled sodium cholesterol sulfate was injected into a female patient who had an inoperable carcinoma of the left adrenal gland and who was excreting over 150 mg of 17-ketosteroids in her urine per day. In an attempt to delay its growth, the tumor was perfused *in situ* with the antimetabolite, 5-fluorouracil. For this purpose a small catheter was introduced under radiographic visualization (via the femoral artery) into the left splenic artery, the vessel supplying 90% of the blood to the tumor. Immediately prior to the introduction of the antimetabolite, 2.5 mg of sodium cholesterol-7α-[^{3}H] [^{35}S]sulfate (24.9×10^6 cpm ^3H; 4.35×10^6 cpm ^{35}S; $^3H/^{35}S$ ratio = 5.7), dissolved in 1 ml of methanol and 50 ml isotonic saline was injected through the catheter. Urine was collected for 3 days in separate daily batches.

Isolation of Metabolites.—The urine specimen of day 1 was first saturated with $(NH_4)_2SO_4$ and the conjugates extracted into a mixture of ether and ethanol (3:1) according to the method of Edwards *et al.* (1952). The organic solvents were removed by distillation and the aqueous residue was extracted with 1-butanol. After the 1-butanol extract was washed with water and the solvent removed, a residue weighing 1.8 g and containing 585,000 cpm ^3H and 115,000 cpm ^{35}S was obtained. This residue was then chromatographed on 250 g of celite using system C (see Fig. 1). The radioactivity appeared over a wide range comprising holdback volumes 3–7 (I in Fig. 1). The combined fractions weighed 700 mg and contained 3.6×10^5 cpm ^3H and 6.6×10^4 cpm ^{35}S.

In addition, a fraction containing 113,000 cpm ^3H and 21,000 cpm ^{35}S was obtained by washing the column

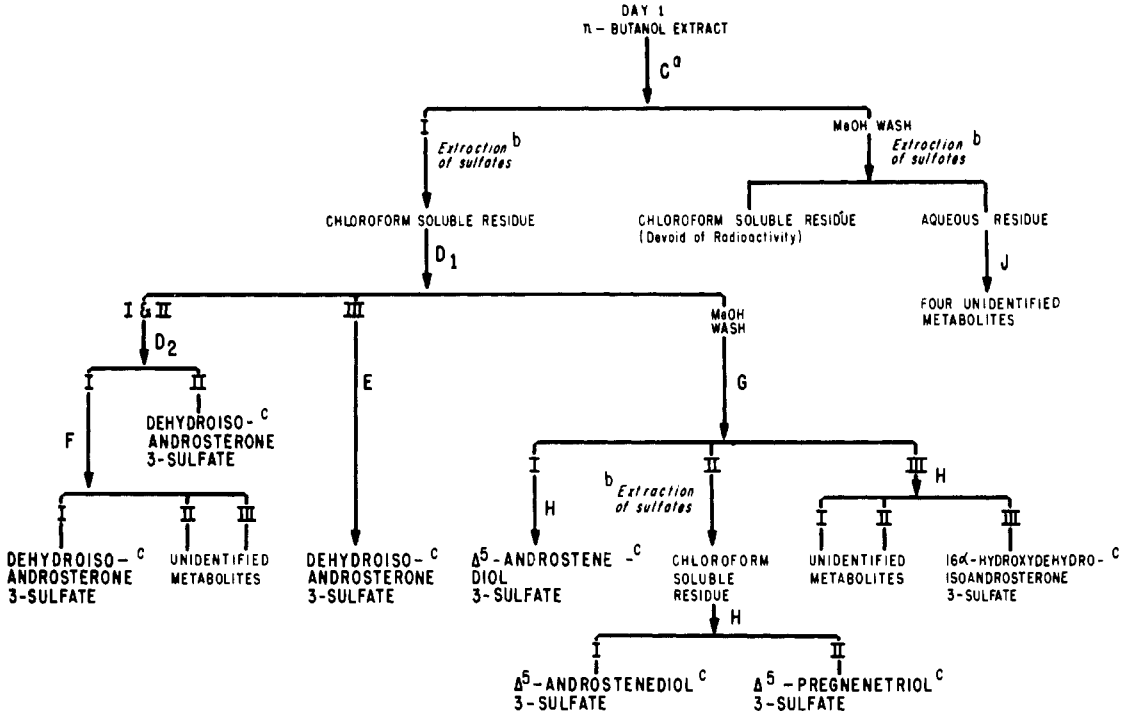

FIG. 1.—Flow sheet for the isolation of metabolites of cholesterol sulfate. [a] Letters refer to celite partition chromatographic systems (see Table I). [b] Extraction of sulfates according to procedure of McKenna and Norymberski (1960); see text for details. [c] Isolated as the ammonium salt.

TABLE II
CRYSTALLIZATION DATA OF DEHYDROISOANDROSTERONE SULFATE ISOLATED FROM URINE SPECIMEN OF DAY 1

Crystallization	Wt (mg)	Crystals: Specific Activity ^3H	Crystals: Specific Activity ^{35}S	Crystals: Ratio $^3H/^{35}S$	Residue in Mother Liquor: Specific Activity ^3H	Residue in Mother Liquor: Specific Activity ^{35}S	Residue in Mother Liquor: Ratio $^3H/^{35}S$
1	52	1134	212	5.3	970	174	5.6
2	30	1154	210	5.5	1090	200	5.5
3	18	1141	204	5.6	1155	213	5.4

with methanol. This radioactive material could not be extracted from a 0.3 M pyridinium sulfate solution by $CHCl_3$. The polar material was removed from the pyridinium sulfate solution by extraction into butanol. The butanol extract was concentrated to dryness and the residue was chromatographed on system J. Four minor components were isolated, each of which was methylene blue–positive and contained both isotopes. Unfortunately, none of these conjugates was present in sufficient quantity or purity to permit identification. The conjugates were solvolyzed and the infrared spectrum of each product was determined. The spectra were, however, not identified.

The material present in holdback volumes 3–7 was dissolved in a 0.3 M pyridinium sulfate solution to convert it into the pyridinium salt. This salt was recovered from the aqueous solution by extraction with 2 volumes of chloroform. Evaporation of the $CHCl_3$ left a residue which weighed 350 mg and contained 264,000 cpm ^3H and 39,000 cpm ^{35}S. The aqueous phase was discarded.

The chloroform-extractable residue was chromatographed on 100 g of celite using system D. Three radioactive zones, all methylene blue– and Zimmermann-positive, were eluted. The two less polar materials were poorly resolved. The material in the third zone contained the major portion of the radioactivity (127,000 cpm ^3H and 17,000 cpm ^{35}S) and weighed 136 mg. It was purified by chromatography on 150 g of celite using system E. A single peak of crystalline radioactive material (93,000 cpm ^3H, 17,000 cpm ^{35}S) was eluted in holdback volumes 3–5. After three successive recrystallizations from methanol-acetone a product, melting at 203–206°, was obtained. The specific activities of the three crystalline products and of the material remaining in the mother liquors are shown in Table II. On admixture of the final product with authentic ammonium dehydroisoandrosterone sulfate, no depression of the melting point was observed. The infrared spectra of the isolated product and the standard were identical. The infrared spectra of the ammonium, sodium, and pyridinium salts of dehydroisoandrosterone sulfate, all determined in KBr, are shown in Figure 2.

Ammonium Dehydroisoandrosterone Sulfate-17-oxime.—Twenty mg of the isolated ammonium dehydroisoandrosterone sulfate was dissolved in 1 ml of absolute ethanol containing 40 mg of $NH_2OH \cdot HCl$ and 0.5 ml pyridine and the solution left overnight at room temperature. The reaction mixture was then poured into 10 volumes of 7 M NH_4OH and the aqueous solution extracted with 1-butanol. The organic phase was washed with water and concentrated to dryness. The recovered residue (17 mg) was chromatographed on 20 g of celite using system G. The oxime, eluted in holdback volumes 2–3, appeared as a symmetrical radioactive peak which was characterized by a constant $^3H/^{35}S$ ratio (5.3) over eight peak fractions. The

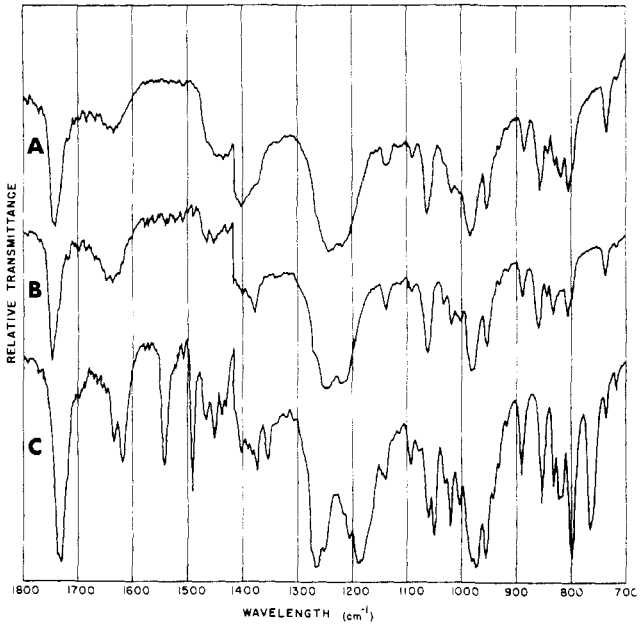

FIG. 2.—Infrared spectra of dehydroisoandrosterone sulfate in KBr. A, Ammonium salt; B, sodium salt; C, pyridinium salt.

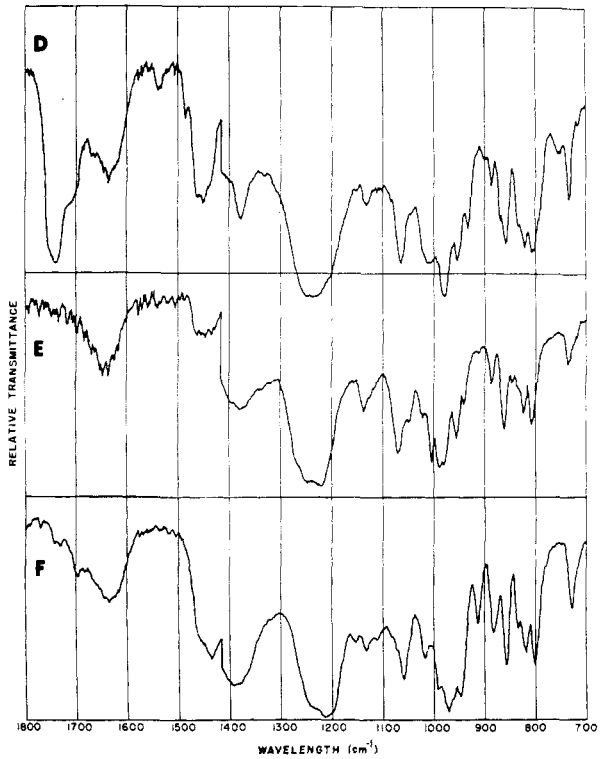

FIG. 3.—Infrared spectra of isolated metabolites in KBr. D, Potassium 16α-hydroxydehydroisoandrosterone-3-monosulfate; E, ammonium Δ^5-androstene-3β,17β-diol-3-monosulfate; F, ammonium Δ^5-pregnene-3β,17α,20α-triol-3-monosulfate.

specific activity of the oxime with respect to both ^3H (1165 cpm/mg) and ^{35}S (208 cpm/mg) was in good agreement with that of the isolated ketone. The infrared spectrum of the oxime was identical with that of authentic ammonium dehydroisoandrosterone sulfate-17-oxime.

The two less polar areas which were poorly separated in the previous chromatogram (D_1 I and II, Fig. 1) were combined and rechromatographed on 50 g of celite using system D. The more polar of the two resulting peaks of radioactivity was identified as an additional quantity of ammonium dehydroisoandrosterone sulfate after treatment with NH_4OH. Further purification was not attempted. The less polar area was rechromatographed on 20 g of celite using system F. A further quantity (3.6 mg) of ammonium dehydroisoandrosterone sulfate was isolated having the same specific activity as the bulk of the ammonium dehydroisoandrosterone sulfate isolated previously. Two more polar radioactive zones were eluted only after the remaining mobile phase was diluted with one-tenth its volume with butanol. The identity of these compounds, both of which gave a positive Zimmermann reaction, is unknown. The less polar peak contained 11,000 cpm ^3H while the more polar peak contained 3000 cpm ^3H. No radioactive sulfur was found in either fraction.

The material eluted from column D_1 by methanol was rechromatographed on 100 g of celite using system G. Three incompletely separated peaks of radioactivity were located. The least polar material (I) was rechromatographed on 20 g of celite using system H. The isolated compound weighed 6 mg and was identified by its infrared spectrum as Δ^5-androstene-3β,17β-diol-3-monosulfate (see below).

The fractions comprising area II (152 mg) were combined and treated with NH_4OH to prepare the ammonium salt. Upon concentration to dryness, there remained a crystalline contaminant (long needles) which was removed by sulbimation at 100° *in vacuo*. The sublimate (109 mg) melted at 75–82° and was nonradioactive and methylene blue–negative. The nonvolatile residue, weighing 43 mg, was dissolved in a 0.3 M pyridinium sulfate solution and extracted into $CHCl_3$. The organic solvent extracted a mixture of sulfates labeled with 30,000 cpm ^3H and 5000 cpm ^{35}S. The mixture was rechromatographed on 50 g of celite using system H. Two methylene blue–positive substances were eluted: compound I in holdback volumes 2.5–3 and compound II in holdback volumes 5–7. Each was recovered as its ammonium salt.

Compound I weighed 9 mg and contained 10,000 cpm ^3H and 1800 cpm ^{35}S. By crystallization from ethanol-ether and then from methanol-acetone, 2 mg of a product having a melting point of 206–209° was obtained. Its specific activity was 1200 cpm/mg with respect to ^3H and 192 cpm/mg with respect to ^{35}S. Its infrared spectrum, shown in Figure 3, was identical with that of authentic ammonium Δ^5-androstene-3β,17β-diol-3-monosulfate, which was prepared by sodium borohydride reduction of authentic ammonium dehydroisoandrosterone sulfate in the following manner: 50 mg of sodium borohydride was added to a solution of 50 mg of sodium dehydroisoandrosterone sulfate in 5 ml of 80% ethanol. The reaction mixture was left overnight at room temperature. Acetone was added to destroy the excess reagent. The solvent was evaporated and the remaining aqueous solution, after the addition of NH_4OH for conversion to the ammonium salt, was extracted twice with 1-butanol. The organic extract was washed with water and concentrated to dryness. The residue was crystallized twice from methanol-acetone-ether and yielded 25 mg of the diol sulfate, melting at 210–213°.

Compound II, weighing 22 mg and containing 20,000 cpm ^3H and 3900 cpm ^{35}S, was isolated as a yellow oil. Most of the yellow color was removed by preliminary leaching with acetone. Crystallization of the residue from ethanol-ether followed by recrystallization from methanol-acetone yielded 4 mg of needles, melting at 179–181°. This compound, whose spectrum is shown in Figure 3, was identified as ammonium Δ^5-pregnene-3β,17α,-

20α-triol-3-monosulfate. The specific activity of the crystalline sulfate was 1120 cpm/mg with respect to ^{3}H and 230 cpm/mg with respect to ^{35}S.[2] A portion of this material (5 mg) was solvolyzed using the method of Burstein and Lieberman (1958). Crystallization of the reaction product from acetone-isooctane yielded 2 mg of pregnenetriol, melting at 221–224° (an authentic sample, generously provided by Dr. H. Hirschmann, melts at 219–222°). The triol was acetylated and the product was crystallized from methanol-acetone, mp 203–206° (authentic Δ⁵-pregnene-3β,17α,20α-triol diacetate melts at 204–206°). Identity was confirmed by its infrared spectrum determined in CS_2. Its specific activity was 1280 cpm/mg with respect to ^{3}H.[2]

Three mg of the isolated Δ⁵-pregnene-3β,17α,20α-triol-3-monosulfate, dissolved in 1.5 ml of methanol, was oxidized in the following manner: A 0.4-ml aliquot of oxidizing solution ($HIO_4 \cdot 2H_2O$ 226 mg, pyridine 0.1 ml, diluted to 10 ml with H_2O) was added and the reaction mixture was left at room temperature overnight. Five ml of water was added and the organic solvents were removed by evaporation. The aqueous residue was made 0.3 M with respect to pyridinium sulfate and then extracted twice with chloroform. The chloroform-soluble residue exhibited an infrared spectrum (CH_2Cl_2) identical with that of authentic pyridinium dehydroisoandrosterone sulfate. The product was converted to its ammonium salt and its specific activity was found to be 1154 cpm/mg with respect to ^{3}H and 254 cpm/mg with respect to ^{35}S (using the methylene blue reagent as a measure of weight).

The material present in zone III of the chromatogram using system G was rechromatographed on 20 g celite using system H. Three areas of methylene blue–positive material were located. The least polar of these three substances weighed less than 150 μg and in CH_2Cl_2 solution exhibited infrared absorption bands at 1750 and 1675 cm^{-1}. The conjugate was solvolyzed and the product was found to possess absorption bands (CS_2) at 1748 and 1675 cm^{-1} in the carbonyl region. Its spectrum in the fingerprint region could not be associated with that of any steroid available to us.

The second sulfate of intermediate polarity was also solvolyzed and the hydrolyzed product was found to possess infrared absorption bands (CS_2) at 1740 and 1720. Its spectrum also remains unidentified.

The most polar conjugate (holdback volume 11) weighed 2 mg. It too was cleaved by solvolysis and the product exhibited an infrared spectrum identical with that of 16α-hydroxydehydroisoandrosterone.[3] As a result, the sulfated metabolite is assumed to be the 3-sulfate of this C_{19}-ketol. The infrared spectrum of its potassium salt is shown in Figure 3. By the time this compound was isolated, considerable decay of the radioactive sulfur had occurred and this made accurate counting difficult. The corrected specific activities of this compound are not recorded because the error associated with them, due to the low level of radioactivity, was large. Nevertheless, the $^{3}H/^{35}S$ ratio was essentially the same as that of the isolated dehydroisoandrosterone sulfate when correction was made for the decay of the ^{35}S.

The urine specimens of the second and third days were processed in the manner previously described for the specimen of day 1. In these instances, however, no effort was made to isolate any conjugate other than dehydroisoandrosterone sulfate. The specific activities of this compound as well as its $^{3}H/^{35}S$ ratio are given in Table III.

TABLE III
DEHYDROISOANDROSTERONE SULFATE DATA

Day	Urinary Excretion mg/24 hr	Final Specific Activity (cpm/mg) ^{3}H	Final Specific Activity (cpm/mg) ^{35}S	Ratio $^{3}H/^{35}S$
1	150	1141	204	5.4
2	85	1550	370	4.2
3	30	1710	188	9.1

DISCUSSION

Evidence has been presented which adds further support to the concept that sulfated intermediates are involved in some of the biosynthetic pathways used for the formation of the steroid hormones. Until now, such conjugates were implicated in only those biosynthetic steps which pertained to the conversion of the C_{21} steroids (e.g., pregnenolone) to C_{19} steroids (e.g., dehydroisoandrosterone). In this report, we have shown that sulfates may also be involved in the transformations that antecede the first C_{21} steroid in the biogenetic sequence, i.e., they may be intermediates in reactions by which the C_{27} sterol, cholesterol, is converted to pregnenolone. From this investigation it is now evident that at least the following processes can use 3β-sulfoxy-Δ⁵-steroids as reactants: 20α-hydroxylation, 22-hydroxylation, and presumably cleavage between C-20 and C-22 (i.e., cholesterol sulfate → pregnenolone sulfate); 17α-hydroxylation and cleavage between C-17 and C-20 (i.e., pregnenolone sulfate → dehydroisoandrosterone sulfate); 16α-hydroxylation (i.e., dehydroisoandrosterone sulfate → 16α-hydroxydehydroisoandrosterone sulfate); reduction of the C-20 carbonyl group (i.e., 17α-hydroxypregnenolone sulfate → Δ⁵-pregnenetriol sulfate); and reduction of the C-17 carbonyl group (i.e., dehydroisoandrosterone sulfate → androstenediol-3-sulfate).

Since these conversions were first shown to occur with 3β-hydroxy-Δ⁵-steroids, it has generally been assumed that enzymes catalyzing the transformation of C_{27} intermediates to C_{19} compounds used hydroxylated as opposed to sulfated steroids as substrates. Since enzymes might normally be expected to differentiate between reactants of such different polarities, it would seem reasonable to postulate the existence of a series of enzymes which prefer the sulfates as substrates. On the other hand, if only one set of enzymes is actually present, it is conceivable that the physiological substrate for at least some of the enzymes are sulfates. There is a suggestion that this may be true in the subject studied in the present experiment. The isolation of a 17α-hydroxylated C_{21} urinary metabolite (pregnenetriol sulfate) whose specific activities with respect to both ^{3}H and ^{35}S are approximately equal to those in two C_{19} metabolites (dehydroisoandrosterone sulfate and androstenediol-3-sulfate, see Table IV) implies that the routes by which radioactive cholesterol sulfate has been converted to the radioactive metabolites may be essentially the same as those which lead to the endogenously formed compounds. The fact that dehydroisoandrosterone sulfate, Δ⁵-androstenediol sulfate, and pregnenetriol sulfate have the same specific activities with respect to both isotopes indicates, moreover, that

[2] For comparison, these values have been corrected for the difference between the molecular weight of this compound and that of ammonium dehydroisoandrosterone sulfate.

[3] The infrared spectrum of this compound was identified by Dr. and Mrs. T. F. Gallagher.

they have a common precursor which is sulfated. In contrast, the contribution from 3β-hydroxy-Δ⁵-precursors to these compounds probably is small because were this not so it is unlikely that their specific activities with respect to ^{3}H and ^{35}S would agree as well as they do. Therefore the results suggest that in the subject of this experiment the isolated C_{21} and C_{19} steroidal sulfates are synthesized predominantly through sulfated intermediates using cholesterol sulfate as a precursor. This concept is further supported by the recent isolation

TABLE IV
ISOTOPIC DATA OF METABOLITES ISOLATED FROM URINE SPECIMEN OF DAY 1

Isolated Metabolite	Specific Activity (cpm/mg) ^{3}H	Specific Activity (cpm/mg) ^{35}S	Ratio $^{3}H/^{35}S$
Dehydroisoandrosterone sulfate	1141	204	5.6
Δ⁵-Androstene-3β,17β-diol-3-monosulfate	1200	192	6.2
Δ⁵-Pregnene-3β,17α,20α-triol-3-monosulfate	1120	230	4.9

in this laboratory (Drayer *et al.*, 1964) of cholesterol sulfate from bovine adrenals, demonstrating for the first time that this compound occurs naturally.

The recovery from the first day's urine specimen of metabolites (dehydroisoandrosterone sulfate, Δ⁵-androstenediol sulfate, 16α-hydroxydehydroisoandrosterone sulfate, and Δ⁵-pregnenetriol sulfate) having approximately the same $^{3}H/^{35}S$ ratio as that of the injected tracer has provided excellent evidence of the direct conversion of doubly labeled cholesterol sulfate to doubly labeled dehydroisoandrosterone sulfate. This reaction probably occurred immediately after injection when the tracer passed through the tumorous steroidogenic tissue for the first time. The labeled cholesterol sulfate which escaped into the general circulation without conversion to other steroidal sulfates would be subjected to a variety of processes, one of which could result in separation of the two isotopes (cholesterol sulfate cleaved to tritiated cholesterol and [^{35}S]sulfate ions). These labeled products could now be used for further steroid sulfate synthesis. Thus the $^{3}H/^{35}S$ ratios present in dehydroisoandrosterone isolated from the second and third days' urine specimens are the end results of a complex sequence of events which depend upon such factors as the size of the available dehydroisoandrosterone and sulfate pools (the steroidogenic capacity of the tumor was markedly impaired during this time by the infusion of 5-fluorouracil and this probably resulted in a diminution of the size of the dehydroisoandrosterone pool) and the rates of numerous processes (cleavage, sulfation, reactions involved in the conversion of C_{27} compounds to C_{19} products, and the excretion of dehydroisoandrosterone sulfate and of inorganic sulfate). Since the magnitude of most of these factors is unknown it is obviously difficult to rationalize the observed ratios. Nevertheless, the fact that four urinary metabolites isolated from the urine of day 1 had the same $^{3}H/^{35}S$ ratio, which in turn was identical with that of the injected tracer, suggests that the reaction sequence, cholesterol sulfate → pregnenolone sulfate → 17-hydroxypregnenolone sulfate → dehydroisoandrosterone sulfate, was the predominant source of the radioactivity associated with those metabolites excreted in day 1 and that routes involving cleavage of labeled steroidal sulfates to ^{3}H-labeled free steroids and ^{35}S-labeled inorganic sulfate provided only the minor part of this radioactivity.

The possibility of the existence of two pools of liver cholesterol, one of which is "active" and the other "inactive" metabolically, has been suggested by Rittenberg and Price (1952). Saba and Hechter (1955), as an explanation of their results with adrenal tissue, have also concluded that two metabolic pools of cholesterol must exist, only one of which is active in corticosteroidogenesis. It is probable that at least a portion of this "active cholesterol" exists in the form of its sulfated ester, since this conjugate has now been shown to exist in nature and to serve as a biosynthetic intermediate. These findings expose a hitherto unknown aspect of the biochemistry of cholesterol.

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