Evidence that Steroid Sulfates Serve as Biosynthetic Intermediates. II. *In Vitro* Conversion of Pregnenolone-^{3}H Sulfate-^{35}S to 17α-Hydroxypregnenolone-^{3}H Sulfate-^{35}S*

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Pregnenolone-^{3}H sulfate-^{35}S was incubated with a homogenate of hyperplastic adrenal tissue. The product isolated in greatest yield (>5%) was 17α-hydroxypregnenolone-^{3}H sulfate-^{35}S which bore both isotopes in the same ratio as in the substrate. Radiochemical purity of the product was proved by crystallization with authentic ammonium 17α-hydroxypregnenolone sulfate and by conversion to 17α-hydroxypregnenolone, pyridinium dehydroisoandrosterone sulfate, and dehydroisoandrosterone. This finding confirms and extends the conclusion drawn previously that steroid sulfates may serve as intermediates in biosynthetic reactions.

Recently, evidence has accumulated which indicates that dehydroisoandrosterone sulfate[1] is a secretory product of human adrenals. Baulieu (1962) showed that the concentration of this sulfate in the adrenal venous blood of a patient with an adrenal tumor was greater than that in the peripheral blood. He also reported its isolation from excised tumor tissue. Furthermore, the synthesis of dehydroisoandrosterone sulfate from dehydroisoandrosterone by adrenal tumor homogenates has also been demonstrated (Wallace and Lieberman, 1963; Cohn *et al.*, 1963; Migeon, 1963). Additional evidence for the secretion of dehydroisoandrosterone sulfate in humans was obtained during attempts to measure the secretory rate of free dehydroisoandrosterone (Vande Wiele *et al.*, 1962). Following the injection of labeled dehydroisoandrosterone into normal subjects, the dehydroisoandrosterone sulfate excreted in urine was often found to have a lower specific activity than did several other metabolites of dehydroisoandrosterone. One explanation of this result is that dehydroisoandrosterone sulfate itself is a secreted precursor of urinary dehydroisoandrosterone sulfate. Subsequently, the secretory rate of dehydroisoandrosterone sulfate in normal individuals was estimated in experiments in which dehydroisoandrosterone-^{14}C and dehydroisoandrosterone-^{3}H sulfate were simultaneously administered to the subjects and the specific activities with respect to each label of urinary dehydroisoandrosterone sulfate and dehydroisoandrosterone glucuronoside were compared (Vande Wiele *et al.*, 1963). The results indicated that dehydroisoandrosterone sulfate is one of the more abundant steroids secreted by human adrenals.

Two biosynthetic pathways leading to the formation of dehydroisoandrosterone sulfate have already been uncovered. Mention has been made of the fact that dehydroisoandrosterone can be sulfated by adrenal tissue. More recently we have presented evidence which demonstrated that dehydroisoandrosterone sulfate may be directly synthesized from another sulfated steroid (Calvin *et al.*, 1963). Pregnenolone sulfate-^{35}S was injected into a women with virilizing adrenal metastases and from her urine ^{35}S-labeled dehydroisoandrosterone sulfate was isolated. Although the yield of the conversion was small, we believed the result revealed the existence of biosynthetic pathways involving steroid sulfates as intermediates. In fact, it was pointed out that a larger yield could hardly have been expected from such an *in vivo* experiment.

To extend these observations to *in vitro* conditions and to eliminate even the remote possibility that the ^{35}S-labeled sulfate group had been cleaved from the precursor, pregnenolone sulfate-^{35}S, and had reconjugated with free dehydroisoandrosterone, the experiment reported in this paper was carried out. Pregnenolone sulfate labeled with both ^{3}H and ^{35}S was incubated with a homogenate of hyperplastic adrenal tissue. The formation of dehydroisoandrosterone sulfate or of other steroid sulfates bearing both labels in the same ratio as that present in the substrate would provide rigorous evidence that pregnenolone sulfate could be metabolized by adrenal tissue via pathways involving intact steroid sulfates as intermediates. Several radioactive fractions were obtained by chromatographic analysis of the incubation products. In one of these fractions, 17α-hydroxypregnenolone sulfate, bearing both ^{3}H and ^{35}S in the same ratio as that present in the starting material, was found to be the major radioactive product. Thus proof is presented that adrenal tissue is capable of hydroxylating pregnenolone sulfate at C-17.

EXPERIMENTAL

Melting points were determined on a Kofler block and have been corrected. Infrared spectra were obtained with a Perkin-Elmer infrared spectrophotometer in KBr pellets, 1.5 mm in diameter, using a beam-condensing unit. Colorimetric determinations were performed with a Zeiss spectrophotometer (PMQ II).

Radioactivity was measured by liquid scintillation counting using previously described techniques (Calvin *et al.*, 1963). Samples were evaporated to dryness in glass vials (Wheaton Glass Co.) and, when necessary, were dissolved first in a known volume of absolute methanol to which five volumes of phosphor solution were subsequently added. The phosphor solution

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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-sulfoxy-5-androsten-17-one (17-oxo-5-androsten-3β-yl sulfate); pregnenolone = 3β-hydroxy-5-pregnen-20-one; pregnenolone sulfate = 3β-sulfoxy-5-pregnen-20-one (20-oxo-5-pregnen-3β-yl sulfate); 17α-hydroxypregnenolone = 3β,17α-dihydroxy-5-pregnen-20-one; 17α-hydroxypregnenolone sulfate = 3β-sulfoxy-17α-hydroxy-5-pregnen-20-one (17α-hydroxy-20-oxo-5-pregnen-3β-yl sulfate); pregnenediol = 5-pregnene-3β,20α-diol; pregnanediol = pregnane-3α,20α-diol.

TABLE I
SYSTEMS USED FOR CHROMATOGRAPHY

A[a]	Isooctane 3, *t*-butanol 5, 1 M NH_4OH 5
B[b]	Methylcyclohexane 2.5, ethyl acetate 4, 1-butanol 0.8, methanol 2, 1 M NH_4OH 3
C[c]	Skellysolve C 10, methanol 9, water 1
D[d]	Isooctane 7, ethyl acetate 3, methanol 4, water 1
E	Isooctane 4, ethyl acetate 1, methanol 4, water 1
F	Isooctane 10, benzene 5, methanol 10, water 3

[a] Siiteri *et al.* (1963). [b] Calvin *et al.* (1963). [c] Bush (1952). [d] Arcos *et al.* (1964).

contained 0.3% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-(5-phenyl-oxazolyl)-benzene (Pilot Chemicals). Methanol was not added to this solution if the sample was known to be soluble in toluene. However all counting data are corrected to those efficiencies for each isotope which would obtain in a phosphor solution containing 16.7% methanol. To correct both for variations in counting efficiency and for the decay of ^{35}S, samples in which radioactivity was counted under different conditions or on different dates were related by counting in parallel standards prepared in the phosphor solution containing 16.7% methanol. Internal standards were employed to correct for quenching.

Chromatography.—Paper chromatograms were developed on Whatman No. 1 paper. The distance from the origin to the bottom of the paper strip was 45–47 cm. When the eluting solvent was expected to overrun the paper, the bottom edge was tapered and the overflow was collected and assayed for radioactivity. Column-partition chromatography was performed on Celite (Johns-Manville No. 545), using previously described techniques (Kelly *et al.*, 1962). Stationary phase and Celite were packed in a ratio of 1 ml of stationary phase to 2 g of Celite, and the amount of mobile phase retained by the column (holdback volume) was generally about three times that of the packed stationary phase. The chromatography systems used in this investigation are listed in Table I and are referred to in the text by code letter.

Pregnenolone-7α-^3H Sulfate and Pregnenolone Sulfate-^{35}S.—Pregnenolone-7α-^3H (specific activity = 4.5 × 10^7 cpm/mg), whose purification is described elsewhere (Arcos *et al.*, 1964), was converted to pregnenolone-7α-^3H sulfate by treatment with chlorosulfonic acid in pyridine (Calvin *et al.*, 1963). The preparation and purification of pregnenolone sulfate-^{35}S (specific activity = 3.4 × 10^5 cpm/mg) has been previously reported (Calvin *et al.*, 1963). A mixture of pregnenolone sulfate-^{35}S and the pregnenolone-7α-^3H sulfate was chromatographed on Celite in system A. The only peak of radioactivity occurred in holdback volumes 4–6, and contained both labels. In fourteen consecutive fractions of the peak the ratio of ^3H to ^{35}S did not differ from the average value by more than 5%. The material in these fractions, which was apparently radiochemically homogeneous, was combined, and an aliquot thereof was employed in the incubation reported below.

Pyridinium and Ammonium Salts of 17α-Hydroxypregnenolone Sulfate.[2]—17α-Hydroxypregnenolone (1.4 g) was dissolved by heating in 20 ml of dry pyridine. After the solution had cooled to room temperature, 0.4 ml of chlorosulfonic acid in 2 ml of dry pyridine was added. The mixture was stoppered and heated at 70–80° for 15 minutes. It was then allowed to cool to room temperature after which 80 ml of petroleum ether was added. The resulting pyridinium salt was removed by filtration and washed with ethyl ether. The precipitate was recrystallized from methanol, yielding a product which melted at 192–195°. When chromatographed on Celite in system B, it had a mobility approximately equal to that of dehydroisoandrosterone sulfate and therefore appeared to be a monosulfate. Its infrared spectrum showed strong absorption peaks at 1690 cm^{-1}, 1215–1235 cm^{-1}, and 750 cm^{-1}, indicating, respectively, the presence of the 20-ketone, 3-sulfate (Colthup, 1950), and pyridinium functions (Cannon and Sutherland, 1951). As further evidence for its identity, this compound was converted by a sequence of reactions involving reduction with KBH_4 and cleavage of the glycol intermediate with periodate to a product whose infrared spectrum was identical with that of pyridinium dehydroisoandrosterone sulfate. The details of this conversion were identical with those described below for the identification of ^3H- and ^{35}S-labeled 17α-hydroxypregnenolone sulfate.

The ammonium salt of 17α-hydroxypregnenolone sulfate was obtained by dissolving the pyridinium salt in 1 M NH_4OH and extracting the resulting ammonium salt into 1-butanol. Recrystallization from methanol-acetone of the residue left after the butanol was removed by distillation yielded a product melting at 195–198°. The infrared spectrum of this compound possessed absorption peaks at 1690 cm^{-1} and between 1225 and 1250 cm^{-1}, confirming the presence of the 20-ketone and 3-sulfate groups. The absorption band at 750 cm^{-1}, characteristic of the pyridinium group, was absent.

Preparation of Homogenate and Incubation with Tracers.—The incubation was performed with a homogenate made from hyperplastic adrenal tissue removed at surgery from a 64-year-old woman with Cushing's syndrome. Immediately following its removal, the tissue was trimmed of fat and 4 g of the trimmed tissue was then homogenized for 5 minutes at 0° with a Potter-Elvehjem all-glass homogenizer in a medium of the following composition: 10.6 ml of 0.1 M sodium phosphate buffer, *p*H 7.4; 10 ml of 0.90% NaCl; 0.40 ml of 1.15% KCl; 0.10 ml of 2.11% KH_2PO_4; 0.10 ml of 1.86% $MgSO_4$; and 3.0 mg of sodium fumarate. The homogenate was filtered through cheesecloth and the residue was washed with additional medium. The final volume of the filtered homogenate was 30 ml. At this point the following cofactors were added: glucose-6-phosphate, 50 mg; nicotinamide, 20 mg; and 15 mg each of ATP, DPN, and TPN.

A mixture of 115 μg of ammonium pregnenolone-7α-^3H sulfate (5.2 × 10^6 cpm) and 1940 μg of ammonium pregnenolone sulfate-^{35}S (6.6 × 10^5 cpm) ($^3H/^{35}S$ ratio = 7.9) was added to 15 ml of the above homogenate which was then incubated with shaking for 3 hours at 37°.

Isolation and Characterization of Products.—At the end of the incubation period, ten volumes of absolute ethanol were added and the denatured incubate was allowed to remain overnight at 4°. The precipitated proteins were then removed by filtration after which the filtrate, containing ^3H (5.7 × 10^6 cpm) and ^{35}S (7.2 × 10^5 cpm), was evaporated to dryness. The resulting residue was partitioned between petroleum ether and 90% methanol. The residue from the methanol extract was chromatographed on Celite in system B. The results of this chromatogram are plotted in Figure 1. Four radioactive zones were eluted with fourteen holdback volumes of mobile phase. Zone 1 contained a small amount of ^3H-labeled free steroid (*ca.* 1% of total ^3H counts eluted from the column) which probably was liberated from the steroid sulfate during the incubation or subsequent work-up. The

[2] Prepared in this laboratory by Mr. L. Bandi.

contents of zone 2 (^3H = 3.9 × 10^6 cpm and ^{35}S = 4.7 × 10^5 cpm), eluted in the third to fourth holdback volumes, has not yet been examined because its most abundant constituent was probably the substrate, ammonium pregnenolone sulfate. Zone 3, containing ^3H (4.9 × 10^5 cpm) and ^{35}S (6.3 × 10^4 cpm), was eluted in the fifth to eighth holdback volumes and was found to contain chiefly 17α-hydroxypregnenolone sulfate. The radioactive material in zone 4 (^3H = 1.7 × 10^5 cpm, ^{35}S = 2.1 × 10^4 cpm) was eluted in the tenth to fourteenth holdback volumes and this, also, has not yet been analyzed. The column wash contained an additional amount of radioactive material (^3H = 5.4 × 10^4 cpm, ^{35}S = 6.1 × 10^3 cpm).

17α-Hydroxypregnenolone-^3H (After Solvolysis).—A one-fifth aliquot of the material comprising zone 3 was solvolyzed in tetrahydrofuran (Burstein and Lieberman, 1958) and the residue left after removal of the solvent was distributed between chloroform and water. The chloroform-soluble material was chromatographed on paper on a 12-cm-wide strip in system C in parallel with standards of dehydroisoandrosterone, 17α-hydroxypregnenolone, and pregnenediol. The limb bearing the standards was assayed by phosphomolybdic acid staining (Kritchevsky and Kirk, 1952) and the unknown material was scanned for radioactivity with a Vanguard Model 880 automatic scanner. No radioactivity was observed in the zone parallel to dehydroisoandrosterone, which had migrated approximately 20 cm. Instead, the major peak of radioactivity occurred in a region 3.5–7.0 cm from the origin. An additional small peak (*ca.* 5% of the counts) was present at the origin. The material in the zone between 3.5 and 7.0 cm was eluted with methanol, mixed with 220 μg of carrier 17α-hydroxypregnenolone, and rechromatographed for 5 hours on a 10-cm-wide paper strip in system D. A standard of 5-pregnenediol was run in parallel on an adjacent limb. From the center of the radioactive chromatogram, which contained carrier 17α-hydroxypregnenolone, a 2-mm-wide longitudinal strip was cut and assayed with phosphomolybdic acid (Kritchevsky and Kirk, 1952). The limb bearing pregnenediol was also assayed with this reagent. In this way, 17α-hydroxypregnenolone was found to be present in a diffuse zone between 4 and 15 cm from the origin, whereas the pregnenediol was detected in a region between 18 and 21 cm from the origin. The remainder of the radioactive chromatogram was divided horizontally into 2-cm-long sections, each of which was separately eluted with methanol. Radioactive assay of each eluate revealed a diffuse zone extending from the origin to 17 cm. The fractions comprising the radioactive zone were combined and to an aliquot of the pool with a radioactivity of 24,800 cpm was added 40.2 mg of unlabeled 17α-hydroxypregnenolone. The mixture was twice recrystallized from methanol and the specific activity of the crystals and the residues from the mother liquors was determined on weighed samples. The results, shown in Table II, indicate that the radioactivity was associated with 17α-hydroxypregnenolone.

Ammonium 17α-Hydroxypregnenolone-^3H Sulfate-^{35}S.—Since the most abundant product derived from solvolysis of the material in zone 3 appeared to be 17α-hydroxypregnenolone, it was assumed that the major metabolite present therein was the 3-sulfate of that compound. Therefore, 10 mg of unlabeled ammonium 17α-hydroxypregnenolone sulfate was added to a two-fifths aliquot (^3H = 1.86 × 10^5 cpm, ^{35}S = 2.20 × 10^4 cpm) of the material in zone 3 and the product was recrystallized three times from a mixture of methanol and acetone. The specific activity with respect to both isotopes of the crystals and of the residues from the mother liquors was determined. The crystallized products were weighed directly and the weights of the mother-liquor residues were estimated with the methylene blue reagent of Crepy and Rulleau-Meslin (1960) using ammonium 17α-hydroxypregnenolone sulfate as the standard. As shown in Table II, the specific activities with respect to each label remained constant during the final two crystallizations.

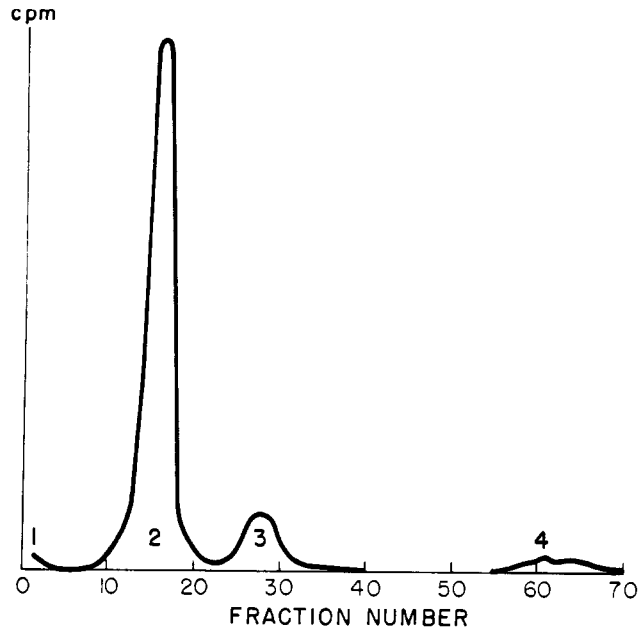

FIG. 1.—Chromatographic analysis of incubation products on Celite in system B. The ordinate records the observed ^3H radioactivity given in arbitrary units. Five fractions are equivalent to one holdback volume.

Conversion to Pyridinium Dehydroisoandrosterone-^3H Sulfate-^{35}S.—Further evidence that the radioactive metabolite was identical with 17α-hydroxypregnenolone sulfate was obtained by conversion of this compound to the pyridinium salt of dehydroisoandrosterone sulfate. The crystals and mother-liquor residue from the final crystallization of 17α-hydroxypregnenolone sulfate were combined (^3H = 7.0 × 10^4 cpm, ^{35}S = 8.4 × 10^3 cpm) and diluted with 13.9 mg of unlabeled ammonium 17α-hydroxypregnenolone sulfate. The mixture was dissolved in 15 ml of absolute ethanol to which 15 ml of water was added. After the addition of 15 mg of KBH_4, dissolved in 2 ml of 0.1 N NaOH, the solution was allowed to remain for 24 hours at room temperature. The reagent was destroyed by adding 1 ml of acetone, after which 15 mg of H_5IO_6, dissolved in 2 ml of NH_4OH, was added to the solution. After 24 hours the excess periodate was consumed by the addition of 0.1 ml of propylene glycol. The resulting solution was evaporated to dryness and the residue was dissolved in 1 M pyridinium sulfate solution. Two extractions with equal volumes of chloroform separated the chloroform-soluble pyridinium salts of steroid sulfates from other water-soluble material (McKenna and Norymberski, 1960). The chloroform extracts were filtered through glass wool and taken to dryness. The resulting residue exhibited an infrared spectrum which was identical with that of the pyridinium salt of dehydroisoandrosterone sulfate. This material was crystallized from a mixture of methylene chloride and acetone and the specific activity of the crystalline product was found to be in good agreement with the predicted value (Table II).

TABLE II
PROOF OF RADIOCHEMICAL PURITY OF COMPOUNDS DERIVED FROM ZONE 3

Compound	Sample	Solvent	Weight (mg)	Specific Activity with Respect to ^3H (cpm/mg)	Specific Activity with Respect to ^{35}S (cpm/mg)	$^3H/^{35}S$ Ratio
17α-Hydroxypregnenolone[a]				6.2×10^{2b}		
	1st crystallization mother liquor	MeOH	35	6.4 7.0	—	—
	2nd crystallization mother liquor	MeOH	26	6.6 6.3	—	—
Ammonium 17α-hydroxypregnenolone sulfate[c]						7.9[d]
	1st crystallization mother liquor	MeOH-acetone	6.5	16.8×10^3 21.5	2.14×10^3 2.51	7.9 8.6
	2nd crystallization mother liquor		4.5	15.7 16.0	1.92 2.04	8.2 7.8
	3rd crystallization mother liquor		2.5	16.5 15.3	1.94 1.81	8.5 8.5
Pyridinium dehydroisoandrosterone sulfate[e]				3.7×10^{3f}	4.4×10^{2f}	
	1st crystallization mother liquor	Methylene chloride-acetone	12	4.0 3.6[g]	4.9 4.7[g]	8.2 7.7
Dehydroisoandrosterone				6.2×10^{3h}	—	—
	1st crystallization mother liquor	MeOH	4.5 1.5	6.1 6.3		

[a] Sample obtained after solvolysis of a one-fifth aliquot of zone 3, followed by two chromatograms on paper; 40.2 mg of unlabeled carrier was added to the radioactive product before crystallization. [b] Predicted value based on the addition of 40.2 mg of carrier to a sample containing tritium (24,800 cpm). [c] Sample obtained by adding 10 mg of carrier ammonium 17α-hydroxypregnenolone sulfate to a two-fifths aliquot of zone 3. [d] Ratio present in the substrate incubated with the homogenate. [e] Sample obtained from doubly-labeled ammonium 17α-hydroxypregnenolone sulfate by treatment with KBH_4 followed by HIO_4; see text for details. [f] Predicted value based on the addition of 13.9 mg of carrier to a 4.3-mg sample containing tritium (7.0×10^4 cpm) and ^{35}S (8.4×10^3 cpm). [g] The preparation of this sample is described in the text. Its specific activity was determined on the ammonium salt but the value reported is that which it would be for the pyridinium salt. [h] Predicted value from the specific activity of the sample's precursor, pyridinium dehydroisoandrosterone sulfate.

Attempts to recrystallize this compound from the same solvent pair were not successful since solution in acetone resulted in solvolysis of the pyridinium steroid sulfate (McKenna and Norymberski, 1957). The purification and identification of the dehydroisoandrosterone produced by this solvolysis is described further on. The instability of the pyridinium salt also caused difficulty when an effort was made to compare the specific activity of the above crystalline pyridinium sulfate with that of the residue left in its mother liquor. Since some solvolysis had occurred in solution, the mother-liquor residue was first freed of unconjugated steroid by treating the residue with 10 ml of 1 M NH_4OH and extracting the resulting mixture three times with equal volumes of methylene chloride. The organic sulfate was then extracted from the aqueous layer with butanol. After being washed once with water, the butanol extract was evaporated to dryness. The esterified sulfate content of the residue was determined by the methylene blue assay (Crepy and Rulleau-Meslin 1960) using ammonium dehydroisoandrosterone sulfate as standard.

The specific activity, determined in this way, was then corrected to the value which would obtain for the pyridinium salt. As shown in Table II, this corrected specific activity was in agreement with that found for the crystalline pyridinium dehydroisoandrosterone sulfate. In addition, the $^3H/^{35}S$ ratios of the crystalline product and of the organic sulfate present in its mother liquor agreed with each other and with the expected ratio.

As mentioned previously, when efforts were made to recrystallize the pyridinium dehydroisoandrosterone sulfate, solvolysis occurred in the acetone solution. From the material left after this inadvertent cleavage tritium-labeled dehydroisoandrosterone was isolated by chromatography on Celite in system E. The radioactive compound, eluted in the fourth holdback volume, was recrystallized from methanol. Its infrared spectrum was identical with that of authentic dehydroisoandrosterone. The specific activity of the crystalline product was determined on a weighed sample and that of the residue left in the mother liquor was estimated on a sample whose dehydroisoandrosterone content was determined by the Zimmermann reaction. As shown in Table II, both values agreed with each other and also with that predicted from the specific activity with respect to ^3H of the precursor, pyridinium dehydroisoandrosterone sulfate.

Presence of Radioactive Dehydroisoandrosterone Sulfate in Zone 3.—A one-fifth aliquot of zone 3 was solvolyzed in tetrahydrofuran (Burstein and Lieberman, 1958) and the residue, after removal of the solvent, was distributed between chloroform and water. To the chloroform-soluble material, which contained ^3H (8.0×10^4 cpm), 10.7 mg of dehydroisoandrosterone was added. The mixture was chromatographed on Celite in system F. In the latter half of the second holdback volume dehydroisoandrosterone was eluted and was found to be radioactive. The compound was recrystallized once from methanol, and then twice from a mixture of petroleum ether and ethyl ether. The specific activities of the three successive crystalline products were, respectively, 1.68, 1.57, and 1.75×10^2 cpm/mg. However those of the residues present in the three mother liquors were higher (5.0, 2.6, and 2.3×10^2 cpm/mg), thus indicating the presence of radioactive impurity. Because of the lack of sufficient material, no further crystallizations were attempted. Nevertheless, assuming that the radioactivity present in the

crystalline products was associated only with dehydroisoandrosterone, it can be calculated that 2% of the tritiated material derived by solvolysis of an aliquot of zone 3 was dehydroisoandrosterone-^{3}H. Since zone 3 contained 9.4% of the ^{3}H incubated, at most 0.2% of the incubated ^{3}H-labeled pregnenolone sulfate could have appeared in this zone as dehydroisoandrosterone-^{3}H-sulfate.

Discussion

Following the incubation of pregnenolone-^{3}H sulfate-^{35}S with a homogenate of hyperplastic adrenal tissue, radioactive 17α-hydroxypregnenolone sulfate was recovered from the incubate in greater than 5% yield. The isolated 17α-hydroxypregnenolone sulfate contained ^{3}H and ^{35}S in the same ratio as that present in the incubated precursor, pregnenolone-^{3}H sulfate-^{35}S. Thus it is possible to conclude unequivocally that the product arose by 17α-hydroxylation of the sulfated substrate.

The conditions of incubation were chosen so that the homogenate contained phosphate ions, which are known (Roy, 1957) to inhibit steroid sulfatase, an enzyme present in adrenal tissue (Ney and Ammon, 1959; Burstein and Dorfman, 1963). Under these conditions it appears that virtually no enzyme-catalyzed cleavage of the doubly labeled pregnenolone sulfate occurred since only trace amounts of radioactivity were found in zone 1 where unconjugated steroids would be expected to occur. In fact, the more polar zones (2–4) contained ^{3}H and ^{35}S in the same ratio as in the starting material. Thus, if other steroid sulfates are present in these fractions, it is likely that they, too, arose only by reactions using steroid sulfates as substrates.

As mentioned under Experimental, pyridinium dehydroisoandrosterone-^{3}H sulfate-^{35}S was inadvertently solvolyzed to dehydroisoandrosterone-^{3}H during attempts at recrystallization from a mixture of methylene chloride and acetone. This result can probably be explained by the findings of McKenna and Norymberski (1957), who found that the pyridinium salt of cholesterol sulfate, when permitted to stand for 4 days in acetone at room temperature, is converted to cholesterol in about 90% yield. Therefore solvolysis of the pyridinium salt of dehydroisoandrosterone sulfate probably resulted from its having been dissolved in acetone for periods of several days.

An intensive effort was made to demonstrate the presence of radioactive dehydroisoandrosterone sulfate among the products of the incubation. This compound was expected to occur in those fractions (zone 3) from which 17α-hydroxypregnenolone sulfate was isolated because the two compounds cannot be resolved in the chromatographic system employed. Solvolysis of an aliquot of zone 3 did in fact yield a radioactive product which appeared to be identical with added carrier dehydroisoandrosterone, although rigorous proof for this was not obtained. However, even if the tritium recovered in this form was associated with dehydroisoandrosterone it would represent a yield of dehydroisoandrosterone-^{3}H sulfate from incubated pregnenolone-^{3}H sulfate of only about 0.2%. Furthermore it cannot be assumed that the radioactive dehydroisoandrosterone sulfate arose via pathways involving only steroid sulfates, since the ^{35}S-labeled sulfate was not shown to be present.

In a previous experiment (Calvin *et al.*, 1963), the *in vivo* conversion of pregnenolone sulfate-^{35}S to dehydroisoandrosterone sulfate-^{35}S in a woman with adrenal metastases was demonstrated. This was considered as evidence that steroid sulfates may serve as biosynthetic intermediates. In particular, it implied the sequence of reactions as shown in Figure 2, in which pregnenolone sulfate is hydroxylated at C-17 and the intermediate ketol is then converted to dehydroisoandrosterone sulfate. This pathway would be analogous to the route by which unconjugated pregnenolone appears to be converted to dehydroisoandrosterone. The finding reported in this paper establishes that the first reaction in the transformation of C_{21}-steroids to to C_{19}-steroids, namely, 17α-hydroxylation, can use a sulfated intermediate as a substrate.

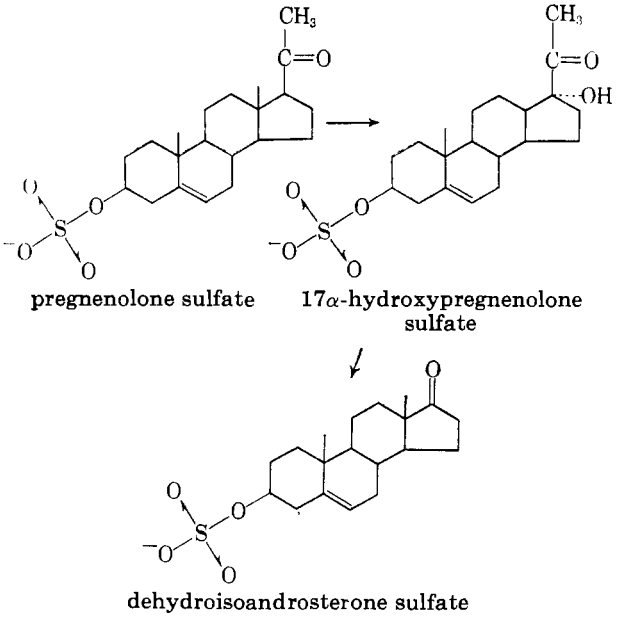

FIG. 2.—Proposed biosynthetic pathway involving steroid sulfates as intermediates.

Relevant to these findings is the evidence that both pregnenolone sulfate and 17α-hydroxypregnenolone sulfate may actually be secreted in humans. Arcos *et al.* (1964) have found, following the administration of pregnenolone-^{3}H to a female subject, that the specific activity with respect to ^{3}H of the urinary pregnenediol was lower than that of the urinary pregnanediol. This result may be taken as support for the notion that pregnenediol arose from a secreted precursor other than pregnenolone. This precursor could have been pregnenolone sulfate, particularly since the work of Gual *et al.* (1962) has suggested that adrenal tissue is capable of converting pregnenolone to pregnenolone sulfate *in vitro*. Moreover, the secretion of 17α-hydroxypregnenolone sulfate by adrenal tumor tissue is likewise implied by the findings of Fukushima *et al.*, (1963), who administered to a female subject 17α-hydroxypregnenolone-^{3}H and isolated from her urine 5-pregnene-3β,17α-20α-triol both after glucuronidase hydrolysis and after acid hydrolysis which was employed to cleave sulfate conjugates. The specific activity with respect to tritium of the triol excreted as the sulfate was found to be less than was that of a sample of triol excreted as its glucuronoside. This result could be explained if 17α-hydroxypregnenolone were secreted by the tumorous adrenals of this subject at least partly as its sulfate.

The observations of Pasqualini and Jayle (1962) are also pertinent to our results. Following the administration of ACTH[3] to normal individuals, these workers were able to isolate from urine the 3,21-disulfate of 3β,21-dihydroxy-5-pregnen-20-one. Although it is ob-

[3] Abbreviation used in this work: ACTH, adrenocorticotropic hormone.

viously not possible to specify where this conjugated metabolite was formed, the presence of the 3β-sulfoxy-5-ene grouping in this compound as well as in pregnenolone sulfate, 17α-hydroxypregnenolone sulfate, and dehydroisoandrosterone sulfate merits attention.

The possible metabolic role that steroid sulfates may play in biosynthetic processes has already been discussed in our previous paper (Calvin *et al.*, 1963).

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Purification and Properties of Bovine Factor V: A Change of Molecular Size During Blood Coagulation*

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The preparation of purified factor V from bovine plasma and bovine serum was achieved through chromatography on Sephadex G-200. Evidence was presented in support of a molecular weight and activity difference between factors V from the two sources. Factor V from plasma was eluted quantitatively as a single peak with a distribution coefficient of 0.05, while factor V from serum was eluted in two separate fractions, one with a distribution coefficient of 0.05 and the other with a distribution coefficient of 0.18. This latter component of activity was present to a significantly greater amount in two-day-old serum than in fresh serum. Density-gradient centrifugation confirmed that the factor V activity from serum with a distribution coefficient of 0.18 had a smaller molecular weight than plasma factor V. Factor V with a distribution coefficient of 0.18 and an apparently smaller molecular weight could also be obtained from purified plasma factor V by treatment with thrombin. This latter technique provided a facile way to the purification of thrombin-activated factor V and resulted in a tenfold increase in activity. It was concluded that thrombin, as a proteolytic enzyme, split the molecule of factor V with the production of a new factor V with a smaller molecular weight.

The classical theory of blood coagulation was formulated at the beginning of the century (Morawitz, 1905) and described the interaction of four factors (thromboplastin, calcium, prothrombin, fibrinogen) with the resultant formation of insoluble fibrin. The first addition to the above four factors was factor V,[1] which was described under different names by three independent groups.

Quick (1943) recognized that there existed another factor necessary for the rapid activation of prothrombin by tissue extract; it was named "labile factor" because of its disappearance in stored oxalated plasma. Later, Owren (1947) published his work based on the discovery of a patient with a hemorrhagic state, but lacking none of the then-recognized clotting factors. He consequently postulated the existence of an additional factor necessary for the activation of prothrombin by tissue extracts, which he initially termed "factor V" and later "proaccelerin" (1950). At approximately the same time, Ware *et al.* (1947a) reported on a substance in bovine plasma which could accelerate the activation of prothrombin in the presence of thromboplastin and calcium, and which they termed

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[1] This name has been adopted by the International Committee on Nomenclature of Blood Clotting Factors (*J. Am. Med. Assoc. 170*, 325, 1959).