tent observed by Minder and Abelin in comparing the 3- and 4-week-old animals may be related to the abrupt dietary change that takes place with weaning (usually at about 3 weeks of age). In contrast, such an abrupt change was avoided in the experiment reported here by allowing the rats to remain with their mothers.

If, as present evidence suggests, plasmalogens are specifically localized in cell membranes, then the relatively constant phospholipid pattern implies that there is no further differentiation in terms of formation of membrane material (cellular or subcellular) in such tissues as heart, lung, liver, and spleen during the period from 1 to 8 weeks.

The spectrophotometric iodination method for measuring α,β-unsaturated ethers, originally developed to characterize relatively pure phosphatide mixtures (Gottfried and Rapport, 1962), has been found to be quite useful for analysis of crude lipids when only very small amounts of sample are available. For example, in the pooled spleens of 1-week-old rats, the entire sample contained only 0.8 μmole of α,β-unsaturated ether. Values for the molar ratio of I_2/NPH obtained with this method with extracts of various rat tissues other than liver agree well with those previously reported by Rapport and Lerner (1959) and Norton (1960). In the case of rat liver lipids, the average value found in this study was 0.65, which is close to that recorded by Norton (0.71) and much lower than values reported by Rapport and Lerner (0.90). This discrepancy has recently been found by Camejo *et al.* (1963) to result from interference by vitamin A in crude extracts.

References

Bieth, R., Freysz, L., and Mandel, P. (1961), *Biochim. Biophys. Acta 53*, 576.

Camejo, G., Rapport, M. M., and Morrill, G. (1963), in preparation.

Erickson, N. E., and Lands, W. E. M. (1959), *Proc. Soc. Exp. Biol. Med. 102*, 512.

Folch, J., Ascoli, I., Lees, M., Meath, J. A., and Le Baron, F. N. (1951), *J. Biol. Chem. 191*, 833.

Gottfried, E. L., and Rapport, M. M. (1962), *J. Biol. Chem. 237*, 329.

Gottfried, E. L., and Rapport, M. M. (1963), *J. Lipid Res. 4*, 57.

Korey, S. R., and Orchen, M. (1959), *Arch. Biochem. Biophys. 83*, 381.

Minder, W. H., and Abelin, I. (1954), *Z. Physiol. Chem. 298*, 121.

Norton, W. T. (1960), *Biochim. Biophys. Acta 38*, 340.

Rapport, M. M., and Franzl, R. E. (1957), *J. Neurochem. 1*, 303.

Rapport, M. M., and Lerner, B. (1959), *Biochim. Biophys. Acta 33*, 319.

Thiele, O. W., Schröder, H., and Berg, W. V. (1961), *Z. Physiol. Chem. 322*, 147.

Wittenberg, J. B., Korey, S. R., and Swenson, F. H. (1956), *J. Biol. Chem. 219*, 39.

Evidence that Steroid Sulfates Serve as Biosynthetic Intermediates: *In vivo* Conversion of Pregnenolone-Sulfate-S^{35} to Dehydroisoandrosterone Sulfate-S^{35}*

Harold I. Calvin,† Raymond L. VandeWiele,‡ and Seymour Lieberman

From the Departments of Biochemistry and Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University, New York

Received January 21, 1963

Pregnenolone sulfate-S^{35} (NH_4^+ salt) (1.1×10^7 cpm) was administered to a female subject with adrenal cancer who was excreting 300 mg/day of 17-ketosteroids. A tracer amount of tritiated dehydroisoandrosterone sulfate (1.7×10^5 cpm) was added during the processing of the first 24-hour urine collection, and the endogenously produced dehydroisoandrosterone sulfate was isolated by celite partition chromatography. The conjugate was purified by successive recrystallization to a constant H^3/S^{35} ratio (4.9), which was not altered by conversion to the oxime of dehydroisoandrosterone sulfate. Thirteen per cent of the administered S^{35} was extracted by the Edwards-Kellie-Wade procedure from the first day's urine (1.4×10^6 cpm), and of this 2.5% (3.5×10^4 cpm) was recovered as dehydroisoandrosterone sulfate-S^{35}. That pregnenolone sulfate may serve as a direct *in vivo* precursor of dehydroisoandrosterone sulfate reveals a heretofore unsuspected and still undefined metabolic role for sulfate conjugates.

The classical view of steroid hormone metabolism has assumed that the hormones are synthesized as free steroids in endocrine tissue, and then prepared for excretion in urine by peripheral metabolism and conjugation. Recently, however, evidence has been obtained that dehydroisoandrosterone,[1] the chief precursor of the 17-ketosteroids found in urine (Vande-

* Supported in part by USPHS Grant A 110 and Naval Research Grant NONR 266(75). A preliminary report of this work has appeared (Calvin *et al.*, 1963).

† Predoctoral Research Fellow, National Institutes of Health.

‡ Investigator of the Health Research Council of the City of New York.

[1] The following trivial names and abbreviations have been used throughout the text: dehydroisoandrosterone = 3β-hydroxy-5-androsten-17-one; dehydroisoandrosterone sulfate = 3β-sulfoxy-5-androsten-17-one; androsterone = 3α-hydroxyandrostan-17-one; etiocholanolone = 3α-hydroxyetiocholan-17-one; androstenedione = 4-androstene-3,17-dione; testosterone = 17β-hydroxy-4-androsten-3-one; pregnenolone = 3β-hydroxy-5-pregnen-20-one; pregnenolone sulfate = 3β-sulfoxy-5-pregnen-20-one; 17α-hydroxypregnenolone sulfate = 3β-sulfoxy-17α-hydroxy-5-pregnen-20-one; pregnenediol-3-monosulfate = 3β-sulfoxy-5-pregnen-20α-ol.

Wiele and Lieberman, 1960), is secreted by the adrenal gland in part as its sulfate ester. Baulieu (1960) found, in a patient with an adrenal tumor, that the concentration of dehydroisoandrosterone sulfate in adrenal vein blood was higher than that present in peripheral blood, implying that the conjugate was synthesized by the gland. More recently, it was, in fact, demonstrated that a homogenate of a human adrenal tumor was able to convert dehydroisoandrosterone to dehydroisoandrosterone sulfate (Wallace and Lieberman, 1963; Cohn *et al.*, 1962). Furthermore, Gual *et al.* (1962) recently reported that, after incubating a homogenate of a human adrenal with cholesterol-C^{14} and pregnenolone-H^3, it was possible to isolate pregnenolone as well as dehydroisoandrosterone containing both isotopes. Both these steroids appeared to be conjugated, probably as sulfates, since they were extractable from the aqueous medium only after cleavage by solvolysis (Burstein and Lieberman, 1958).

Additional evidence for the secretion of dehydroisoandrosterone sulfate by the adrenal was obtained in this laboratory during attempts to measure the secretory rate of dehydroisoandrosterone (VandeWiele *et al.*, 1962). After the administration of tritiated dehydroisoandrosterone to human subjects, dehydroisoandrosterone itself was isolated from the sulfate fraction of the urine and androsterone and etiocholanolone were isolated from the glucuronoside fraction. Since the latter compounds, androsterone and etiocholanolone, are known to be metabolites of androstenedione and testosterone, as well as of dehydroisoandrosterone, the isolated urinary dehydroisoandrosterone, which could not arise from these two α,β-unsaturated ketones, was expected to bear a higher specific activity than either androsterone or etiocholanolone. However, although the experiment was carried out in more than twenty subjects, this result was never found. Generally, the specific activity of dehydroisoandrosterone was equal to those of androsterone and etiocholanolone, but in many instances, it was considerably lower than those of the other two 17-ketosteroids. This implied that the urinary dehydroisoandrosterone sulfate was derived not only from secreted dehydroisoandrosterone, but also from some other precursor which was excreted as dehydroisoandrosterone sulfate without being first converted to dehydroisoandrosterone. These findings could best be explained by assuming that dehydroisoandrosterone sulfate was itself secreted by the glandular tissue, and that part of it was excreted unchanged into the urine. When that fraction of dehydroisoandrosterone excreted as a glucuronoside was examined it was found (VandeWiele *et al.*, 1963) that its specific activity was much higher than that of dehydroisoandrosterone excreted as sulfate. This further supported the conclusion that urinary dehydroisoandrosterone sulfate is derived, at least in part, from precursors other than dehydroisoandrosterone. In experiments using dehydroisoandrosterone-C^{14} and dehydroisoandrosterone sulfate-H^3, Gurpide *et al.* (1963) were able to estimate the secretory rates of both dehydroisoandrosterone and dehydroisoandrosterone sulfate, and thereby confirmed that dehydroisoandrosterone sulfate is one of the major steroids secreted by human adrenals (VandeWiele *et al.*, 1963). It is evident, therefore, that information about the biosynthetic origin of this sulfate is of considerable importance. There are two general routes which can be envisioned for the biosynthesis of dehydroisoandrosterone sulfate. One has already been demonstrated; adrenal tumor tissue can make dehydroisoandrosterone sulfate from dehydroisoandrosterone (Wallace and Lieberman 1963; Cohn *et al.*, 1962). An alternative pathway could be the direct formation of dehydroisoandrosterone sulfate from another steroid sulfate. This possibility is particularly intriguing since, if it were true, it would imply the existence of a biosynthetic pathway involving steroid sulfates as intermediates.

In this paper, results are presented which appear to confirm the existence of such a pathway. Ammonium pregnenolone sulfate (3β-sulfoxy-Δ^5-pregnen-20-one), labeled with S^{35}, was administered intravenously to a woman with adrenal cancer who was excreting 300 mg/day of 17-ketosteroids. A tracer amount of dehydroisoandrosterone-7α-H^3 sulfate was added during the processing of the first 24-hour urine collection. The endogenously excreted dehydroisoandrosterone sulfate was then isolated and found to contain both H^3 and S^{35}. Since the H^3/S^{35} ratio in the isolated conjugate reached a value which remained constant through a number of purifications, the conclusion may be drawn that pregnenolone sulfate-S^{35} was converted *in vivo* to dehydroisoandrosterone sulfate-S^{35}.

Experimental

Melting points were determined on a Köfler block and have been corrected. Infrared spectra were obtained with a Perkin-Elmer infrared spectrometer (Model 221).

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (Model 314-DC) at high voltage tap 9 (1230 v), using discriminator settings of 10 v to 100 v for determination of H^3, and 100 v to infinity for determination of S^{35}. When both labels were present in a given sample, they were estimated simultaneously at these settings according to the technique of Okita *et al.* (1957). Samples to be counted were dried down in glass vials (Wheaton Glass Co.), and then dissolved in a known volume of absolute methanol. To this solution was added 5 volumes of a toluene solution containing 0.3% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-(5-phenyloxazolyl)-benzene (Pilot Chemicals). To correct both for variations in counting efficiency and for the decay of S^{35}, samples counted on different dates were related by reading them in parallel with standards prepared in the above counting solution. Internal standards were employed to correct for quenching.

Chromatography.—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 (hold-back volume) was generally about three times that of the packed stationary phase. Since a number of chromatography systems were used in this investigation, several of which have been developed during the course of the experiments, these systems are listed in Table I. With the exception of methylcyclohexane (Phillips Petroleum Co., technical grade), the solvents used to prepare these systems either were of analytical grade or were distilled before use.

Ammonium Pregnenolone Sulfate-S^{35}.—At 0°, 0.1 ml of S^{35}-labeled chlorosulfonic acid (New England Nuclear Corp.) was added to 0.5 ml of dry pyridine. A solution of 88 mg of pregnenolone (mp 192–93°), dissolved in 1 ml of dry pyridine, was added at 0° to the tube containing the sulfating reagent. The solution was heated for 1 minute until it became clear, and then it was stoppered and allowed to stand overnight at room temperature. After the addition of 10 vols of 7 M NH_4OH, the reaction mixture was extracted twice with *n*-butanol. The butanol extracts were washed with

TABLE I
SYSTEMS USED FOR CELITE PARTITION CHROMATOGRAPHY

System	Components
A[a]	Isooctane 3, *t*-butanol 5, 1 M NH_4OH 5
B	Methylcyclohexane 2.5, ethyl acetate 4, *n*-butanol 0.8, methanol 2, 1 M NH_4OH 3
C[a]	Isooctane 2, *t*-butanol 5, 1 M NH_4OH 5
D[a]	Isooctane 2.7, *t*-butanol 5, 1 M NH_4OH 5
E	Isooctane 2, ethyl acetate 4, *n*-butanol 1.5, methanol 2, 1 M NH_4OH 3

[a] Siiteri (1963).

water and evaporated to dryness, and the residue chromatographed in system A. A single radioactive peak was observed in hold-back volumes 5–7. The residue obtained by evaporation of the solvent from these fractions was recrystallized from methanol-acetone. The resulting product, ammonium pregnenolone sulfate, melted at 198–201°, and contained 5.5×10^7 cpm of S^{35}. This sample was employed as tracer in the present study. It had a specific activity of 1.22×10^6 cpm/mg. The residue from its mother liquor, containing 6.0×10^7 cpm, had a specific activity of 1.21×10^6 cpm/mg. The infrared spectrum of the crystalline product, determined in KBr, showed a strong absorption band at 1700 cm^{-1}, indicating the presence of a ketone group on C_{20}, and a broad peak between 1215 and 1250 cm^{-1}, which is characteristic of the sulfate ester group (Colthup, 1950). When 3.2×10^5 cpm of the above tracer was mixed with 16 mg of nonradioactive ammonium dehydroisoandrosterone sulfate and the mixture chromatographed on celite in system B, which can separate completely the sulfates of pregnenolone and dehydroisoandrosterone, the only peak of radioactivity appeared in hold-back volumes 2–4. Dehydroisoandrosterone sulfate, recovered from hold-back volumes 7–9 and recrystallized from methanol-acetone, was devoid of radioactivity.

Ammonium Dehydroisoandrosterone-7α-H^3 Sulfate.[2]—Dehydroisoandrosterone-7α-H^3 acetate (specific activity 11.6 mc/mg) (New England Nuclear Corp.) was saponified by heating at reflux temperature for 2 hours in a 5% solution of KOH in methanol. The saponified material was extracted from water into ethyl acetate, and then chromatographed for 16 hours on paper in parallel with standard dehydroisoandrosterone in a system composed of cyclohexane (100), dioxane (25), methanol (100), and water (10). The radioactive tracer was located with an automatic radioactivity scanner (Vanguard Instruments, Model 880) and the major peak of radioactive material, which had the same mobility as standard dehydroisoandrosterone, was eluted from the paper with methanol. The radioactive dehydroisoandrosterone was then sulfated with chlorosulfonic acid in pyridine by the technique described above, and the butanol-extractable products were chromatographed on celite in system A. The single radioactive zone observed was pooled and rechromatographed in the same system. From this last chromatogram a single radioactive substance was recovered and employed in experiments as standard ammonium dehydroisoandrosterone-7α-H^3 sulfate. Its purity was verified as follows: When an aliquot of this tracer was mixed with unlabeled ammonium dehydroisoandrosterone sulfate, the resulting product had a specific activity which remained equal in crystals and mother liquor residues during two recrystallizations. Chromatography in system A of this mixture revealed identical mobilities for the carrier, as assayed by the procedure of Crepy and Rulleau-Meslin (1960), and for the radioactivity. In addition, when an aliquot of the tracer was solvolyzed (Burstein and Lieberman, 1958) and mixed with carrier unlabeled dehydroisoandrosterone, the resulting product had a specific activity which remained constant in crystals and mother liquor residues during two recrystallizations.

[2] Prepared in this laboratory by Dr. J. J. Cos.

Injection of Pregnenolone Sulfate-S^{35} and Isolation of Dehydroisoandrosterone Sulfate-S^{35} from Urine.—Ammonium pregnenolone sulfate (1.09×10^7 cpm) was administered intravenously to a previously described (Wallace and Lieberman, 1963) female patient. The subject had a metastatic adrenal carcinoma which had recurred after adrenalectomy. At the time of injection she was excreting about 300 mg of 17-ketosteroids per day. After the injection, her urine was collected for 3 days in separate daily batches. From the first day's collection, dehydroisoandrosterone sulfate was isolated as follows: The urine was first saturated with $(NH_4)_2SO_4$, and the steroid conjugates were then extracted with a mixture of ether and ethanol (3:1) following the procedure of Edwards *et al.* (1952). The organic layer, containing 1.4×10^6 cpm, was concentrated, filtered to remove $(NH_4)_2SO_4$, and evaporated to dryness. The resulting residue (5.6 g) was chromatographed on celite (240 g) in system C. Elution with 7 hold-back volumes of mobile phase resulted in two incompletely separated peaks of radioactivity. The less polar peak occurred in the fractions where steroid monosulfates were expected. Therefore these fractions, containing 8.4×10^5 cpm, were combined for the isolation of dehydroisoandrosterone sulfate. The more polar peak (4.6×10^5 cpm), located where steroid disulfates have been found, was saved for subsequent examination.

The residue obtained from those fractions which were presumed to contain monosulfates (3.5 g) was redissolved in 30 ml of 0.3 M pyridinium sulfate and the resulting solution was extracted successively with 15-ml and 30-ml portions of chloroform. The pyridinium salt of dehydroisoandrosterone sulfate is almost quantitatively extracted into chloroform by this procedure (McKenna and Norymberski, 1960). The residue (1.3 g) left after removal of the chloroform from the combined extracts contained 6.1×10^5 cpm of S^{35}. To it 1.72×10^5 cpm of ammonium dehydroisoandrosterone-7α-H^3 sulfate was added, and the mixture chromatographed on celite (250 g) in system D. Elution was carried out with 11 hold-back volumes of mobile phase. One area of radioactivity, which contained both H^3 and S^{35}, appeared in hold-back volumes 6–10. Since H^3 was unequally distributed in this peak, occurring mainly in the latter portion, it was clear that dehydroisoandrosterone sulfate-S^{35} had not been separated from other S^{35}-labeled materials. Therefore, to facilitate the isolation of dehydroisoandrosterone sulfate, the most polar fractions of the peak, containing 1.0×10^5 cpm of H^3 and 0.4×10^5 cpm of S^{35}, were combined. In them was approximately 60% of the H^3 introduced immediately prior to this chromatography.

When the residue from these fractions (126 mg) was rechromatographed in system B, only one area of radioactivity was observed. It was eluted in hold-back volumes 5–7 and contained both isotopes. All fractions which contained H^3 also contained S^{35}, although the H^3/S^{35} ratio was not constant in successive fractions of the peak. A crystalline residue weighing 117 mg and containing 9.0×10^4 cpm of H^3 and 3.5×10^4 cpm of S^{35} was recovered from the radioactive fractions. Recrystallization of this material from methanol-acetone yielded a product (mp 205–207°) whose infrared spectrum in KBr was identical with that of synthetic ammonium dehydroisoandrosterone sulfate. Four suc-

TABLE II
CRYSTALLIZATION DATA

Sample	H^3/S^{35} Ratio		S^{35}-Specific Activity of Crystals	
	Crystals	Residue in Mother Liquor	cpm/mg	cpm/μmole
Dehydroisoandrosterone sulfate				
1	4.4	1.4	332	128
2	4.7	3.9	315	121
3	4.9	4.6	287	110
4	4.8	4.8	282	108
Dehydroisoandrosterone sulfate oxime				
1	4.8	4.9	250	100
2	5.0	5.0	261	104
Eluates[a]	5.0			

[a] Average of the values determined for seven consecutive fractions of the chromatographic peak.

cessive recrystallizations of the isolated metabolite from methanol-acetone were performed and the ratios of H^3 to S^{35} in crystals and residues from the mother liquors determined. In addition, the specific activity of each crystalline product with respect to S^{35} was measured by determining the radioactivity of weighed samples. As shown in Table II, both these parameters reached a constant value.

Ammonium Dehydroisoandrosterone Sulfate-S^{35}-17-Oxime.—Twenty mg of ammonium dehydroisoandrosterone sulfate, isolated from urine (H^3/S^{35} = 4.7) and derived from the last two sets of crystals and mother liquor residues described in Table II, was dissolved in a solution containing 40 mg of $NH_2OH \cdot HCl$, 0.6 ml of pyridine, and 0.8 ml of absolute ethanol, and allowed to stand overnight at room temperature. After the addition of 10 volumes of 7 M NH_4OH the reaction mixture was extracted twice with *n*-butanol. The butanol extracts were washed with water and evaporated to dryness. The residue obtained was then recrystallized from methanol, and the resulting product recrystallized once more from the same solvent. Both sets of crystals and their mother liquor residues showed the same ratio of H^3 to S^{35} as did the parent compound, ammonium dehydroisoandrosterone sulfate (Table II). The samples of the oxime were pooled and chromatographed in system E, eluting with 8 hold-back volumes of mobile phase. One area of radioactivity in hold-back volumes 4–5 was recovered. The average ratio of H^3 to S^{35} in seven consecutive fractions of this peak was 5.0. In none of these fractions did the ratio differ from this value by more than 10%.

DISCUSSION

The following considerations support the contention that pregnenolone sulfate-S^{35} served as a direct *in vivo* precursor of dehydroisoandrosterone sulfate-S^{35}. The S^{35} radioactivity present in the urinary dehydroisoandrosterone sulfate isolated after chromatography in system B could not have been due to either pregnenolone sulfate-S^{35} or a contaminant present in the injected tracer; for when a mixture of unlabeled dehydroisoandrosterone sulfate and a sample of the administered radioactive material was chromatographed on system B, the dehydroisoandrosterone sulfate was recovered entirely free of radioactivity. Moreover, several criteria were used to show that the endogenously produced dehydroisoandrosterone sulfate was truly labeled with S^{35}. After the addition of tritiated dehydroisoandrosterone sulfate, several steps before the final isolation of this conjugate, the isolated sulfate was recrystallized until the H^3/S^{35} ratio in both crystals and in the residues left in the mother liquors from the crystallizations were constant (Table II). In addition, the specific activities of the crystalline products with respect to S^{35} were also constant through two successive recrystallizations. When the purified sulfate was converted to its 17-oxime, the product possessed the same H^3/S^{35} ratio and the same specific activity with respect to S^{35} as did the dehydroisoandrosterone sulfate-S^{35} from which it was derived (Table II). Furthermore, the H^3 and S^{35} present in the oxime had identical chromatographic mobility in system E. Since dehydroisoandrosterone sulfate is considerably less polar than its oxime, this final criterion has added significance. If the S^{35} had been associated with a nonketonic compound such as pregnenediol-3-monosulfate and had not been separated from dehydroisoandrosterone sulfate by the previous chromatographic purifications, it would have been eluted from this last chromatogram considerably earlier than dehydroisoandrosterone sulfate oxime. From these findings, the association of the S^{35} with the isolated dehydroisoandrosterone sulfate seems to have been established.

The amount of dehydroisoandrosterone sulfate-S^{35} present in the extract at the time that dehydroisoandrosterone sulfate-H^3 (1.72×10^5 cpm) was added can be estimated from the average of the final nine values in Table II (4.9), which represents the ratio of H^3 to S^{35} present in purified dehydroisoandrosterone sulfate. The figure thus calculated (3.5×10^4 cpm) corresponds to 0.33% of the injected S^{35}. For several reasons this yield is more significant than it may appear on the surface. First, the dehydroisoandrosterone sulfate-S^{35} was isolated from an extract of only the first day's urine, which contained 1.4×10^6 cpm or 13% of the injected radioactivity as organic sulfate. Second, Roberts *et al.* (1961), after the administration of tritiated dehydroisoandrosterone sulfate, proved that the dehydroisoandrosterone sulfate conjugate is cleaved *in vivo* and as a result only 15% of the injected radioactivity was recovered in the sulfate fraction of a 4-day urine collection. Their low yield of radioactive steroid sulfate from tritiated dehydroisoandrosterone sulfate suggests that the yield from endogenously produced dehydroisoandrosterone sulfate-S^{35} would be even less since the S^{35} conjugate, once cleaved, cannot be reformed as labeled material. Finally, the conversion of peripherally administered C_{21} steroids to urinary C_{19}-17-ketosteroids is well known to be poor. For example, Solomon *et al.* (1960) obtained a 4% yield of urinary dehydroisoandrosterone following the administration of 17α-hydroxypregnenolone to a patient with adrenal carcinoma. The yield of dehydroisoandrosterone from pregnenolone is even smaller judging from the results of Burstein and Dorfman (1962b). Thus it is evident that under the conditions of our experiment, a yield greater than that observed could hardly have been expected.

The evidence that dehydroisoandrosterone sulfate-S^{35} can be synthesized *in vivo* from pregnenolone sulfate-S^{35} indicates a conversion involving a series of reactions (probably via 17α-hydroxypregnenolone sulfate), which proceed without cleavage of the sulfate ester group (Fig. 1). The alternative possibility, that a significant portion of radioactive sulfate cleaved from the injected tracer could be reesterified with dehydroisoandrosterone, seems much less likely, since the intermediate radioactive inorganic sulfate liberated *in vivo* would undoubtedly be subject to enormous dilution by unlabeled ions. For the results reported in this paper

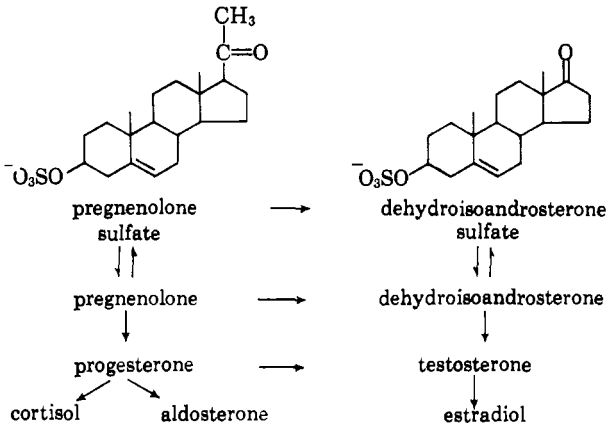

FIG. 1.—Abbreviated scheme of steroid hormone biogenesis illustrating possible involvement of steroid sulfate intermediates.

to have been due to this spurious exchange of S^{35}-sulfate from pregnenolone to dehydroisoandrosterone would require at the very least that this transfer had occurred in the same confined region of the body (e.g., within the adrenal metastases or the liver) where dehydroisoandrosterone sulfate was itself formed since only in this way could the lack of dilution be envisaged. To eliminate even this possibility, experiments using doubly labeled pregnenolone sulfate (S^{35} in the sulfate group and pregnenolone-7α-H^3) as precursor are presently being carried out.

That pregnenolone sulfate may serve as a direct *in vivo* precursor of dehydroisoandrosterone sulfate reveals a heretofore unsuspected and still undefined metabolic role for sulfate conjugates. Sulfate conjugation has already been implicated as a means for "detoxication" of metabolites destined for excretion; however, the significance of the existence of sulfate groups in products such as mucopolysaccharides, sulfatides, and proteins (e.g., tyrosyl-*o*-sulfate, [Bettelheim, 1954]) is unclear. The finding of Roberts *et al.* (1961) that dehydroisoandrosterone sulfate is cleaved *in vivo* suggested that sulfate conjugates are not produced in the liver or kidney merely to facilitate excretion. This was further indicated by the observation that the adrenals and possibly also the other steroid-producing endocrine glands secrete dehydroisoandrosterone sulfate (see above).

It has already been pointed out by Davis (1958) that, while most biosynthetic intermediates are ionic, conspicuous among the exceptions to this generalization were the steroids. In view of the findings reported in this paper, it is entirely possible that the sulfates of at least some steroids serve as ionic, and possibly therefore more active, substrates for certain reactions.

Although the evidence presented here suggests that the *intact* steroid sulfates may serve as substrates in biosynthetic processes, just where the steroid conjugates fit in the scheme of biogenesis is unknown at the present time. In Figure 1 is presented an abbreviated scheme which illustrates how sulfate intermediates might be integrated into previously considered routes of steroid hormone biosynthesis. It is evident from this scheme that the C_{21} steroids, pregnenolone sulfate, pregnenolone, and progesterone, may be considered to reside in a central position, since from them the remaining hormones can be derived. From these intermediates, the diverging stream of biosynthesis can proceed either in the direction of the C_{21} adrenocortical hormones, such as cortisol and aldosterone, or else toward other products, such as testosterone, estradiol, dehydroisoandrosterone, and dehydroisoandrosterone sulfate. The regulatory processes determining which end-products will be formed, and in what quantities, are not yet understood. Obviously, the mechanisms controlling the nature and the amount of the secreted hormones are intimately involved with the enzyme systems responsible for the various conversions along the chain of biosynthesis. Among those systems previously considered are the 3β-hydroxydehydrogenase, the 17β-hydroxydehydrogenase, the enzymes responsible for the cleavage of a ketol side chain, —CHOH—CO—CH_3, to a 17-ketosteroid, the several hydroxylases acting at C_{11}, C_{17}, and C_{21}, and the enzymes concerned with the aromatization of the C_{19} steroids to the C_{18} estrogens. In pathological situations, derangements of one or more of these enzyme systems have been invoked to rationalize the formation of abnormal intermediates and the existence of clinical syndromes. For example, attempts have been made to associate diseases such as congenital adrenal hyperplasia (Eberlein and Bongiovanni, 1960) or ovarian dysfunction (Vande-Wiele, 1960) with defects in some of these enzymatic processes. With the demonstration that 3β-sulfoxy-5-ene intermediates may be involved in steroidogenic processes, the enzymes concerned with the formation of the sulfates or with their cleavage must now also be considered as loci where control may be exerted and where, therefore, derangements may occur. The recent demonstrations (Ney and Ammon, 1959; Burstein and Dorfman, 1962a) that alkyl sulfatases capable of cleaving dehydroisoandrosterone sulfate are present in adrenals support this possibility. The steroid sulfates may serve as a reservoir for the non-conjugated intermediates, in which case the process of sulfation and cleavage will play an important role in the regulation of the rate of synthesis of the steroid hormones.

It is also possible that pathways involving sulfate intermediates are independent of those employing α,β-unsaturated ketones, perhaps by virtue of the localization in the cell or by virtue of zonation in the glands, and that each of these routes possesses different physiologic roles. It is known, for instance, that the rate of production of the androgens by the adrenals varies independently from that of hydrocortisone, and the assumption of pathway for the production of the androgens separate from that leading to the corticosteroids would offer an explanation for this phenomenon. It should also be considered that sulfate pathways may exist, involving as yet undetected intermediates such as the 3β-sulfoxy-5-ene analog of cortisol (3β - sulfoxy - 11,17,21 - trihydroxy - 5 - pregnen - 20-one). Certainly the findings reported in this paper imply that the metabolic capabilities of the endocrine glands will now have to be reevaluated in order to consider the possible roles of such compounds as cholesterol sulfate, pregnenolone sulfate, 17α-hydroxypregnenolone sulfate, and dehydroisoandrosterone sulfate in the biosynthesis of active hormones.

REFERENCES

Baulieu, E. E. (1960), *Compt. Rend. 251*, 1421.
Bettelheim, F. R. (1954), *J. Am. Chem. Soc. 76*, 2838.
Burstein, S., and Lieberman, S. (1958), *J. Biol. Chem. 233*, 331.
Burstein, S., and Dorfman, R. I. (1962a), *Fed. Proc.* (Abstracts) *21*, 248.
Burstein, S., and Dorfman, R. I. (1962b), *Acta Endocrinol. 40*, 188.
Calvin, H. I., VandeWiele, R. L., and Lieberman, S. (1963), *Fed. Proc.* (Abstracts), *22*, 531.

Cohn, G. L., Mulrow, P. J., and Dunne, V. C. (1962), Abstracts of Papers, 44th Meeting of the Endocrine Society, p. 48.
Colthup, J. (1950), *J. Opt. Soc. Am. 40*, 397.
Crepy, O., and Rulleau-Meslin, F. (1960), *Rev. Franc. Etudes Clin. Biol. 5*, 283.
Davis, B. D. (1958), *Arch. Biochem. Biophys. 78*, 497.
Eberlein, W. R., and Bongiovanni, A. M. (1960), *Metab. Clin. Exp. 9*, 326.
Edwards, R. W. H., Kelly, A. E., and Wade, A. P. (1952), *Mem. Soc. Endocrinol. 2*, 53.
Gual, C., Rojo, B., Lemus, A. E., and Rivera, R. (1962), Sociedad Mexicana de Nutricion y Endocrinol., 3rd Meeting, p. 126.
Gurpide, E., MacDonald, P. C., VandeWiele, R. L., and Lieberman, S. (1963), *J. Clin. Endocrinol. Metab., 23*, 346.
Kelly, W. G., Bandi, L., Shoolery, J. M., and Lieberman, S. (1962), *Biochemistry 1*, 172.
McKenna, J., and Norymberski, J. K. (1960), *Biochem. J. 76*, lx.
Ney, K. H., and Ammon, R. (1959), *Z. Physiol. Chem. 315*, 145.
Okita, G. J., Kabara, J. J., Richardson, F., and LeRoy, G. V. (1957), *Nucleonics 15*, 111.
Roberts, K. D., VandeWiele, R. L., and Lieberman, S. (1961), *J. Biol. Chem. 236*, 2213.
Siiteri, P. K. (1963), Ph.D. dissertation, Columbia University, p. 66.
Solomon, S., Carter, A. C., and Lieberman, S. (1960), *J. Biol. Chem. 235*, 351.
VandeWiele, R. L. (1960), *Bull. Sloane Hosp. for Women 6*, 82.
VandeWiele, R. L., and Lieberman, S. (1960), *Biol. Activities Steroids Relation Cancer, Proc. Conf., Vergennes, Vt.*, Pincus, G., and Vollmer, E., eds., New York, Academic, p. 93.
VandeWiele, R. L., MacDonald, P. C., Bolte, E., and Lieberman, S. (1962), *J. Clin. Endocrinol. Metab. 22*, 1207.
VandeWiele, R. L., MacDonald, P. C., Gurpide, E., and Lieberman, S. (1963), *Recent Progr. Hormone Res., 19*, in press.
Wallace, E., and Lieberman, S. (1963), *J. Clin. Endocrinol Metab., 23*, 90.

Blood Estriol Conjugation During Human Pregnancy*

JOSEPH C. TOUCHSTONE, JOHN W. GREENE, JR., ROBERT C. MCELROY, AND TARAS MURAWEC

From the Department of Obstetrics and Gynecology, School of Medicine, University of Pennsylvania, Philadelphia

Received December 7, 1962

Results of estriol determinations and methods used with blood plasma after enzymatic hydrolysis with β-glucuronidase and phenolsulfatase indicated that a large proportion of estriol in the plasma of pregnant women was present as a sulfoglucosiduronate diconjugate (4.5 μg, 44%). The sulfate (3.1 μg, 25%) and a glucosiduronate (3.8 μg/100 ml, 31%) were also present. Cord blood plasma contains estriol mainly as the sulfate (69.0 μg/100 ml, 70%), with smaller amounts as the glucosiduronate (22.0 μg/100 ml, 24%). "Free" ether-extractable estriol averaged 3.7 μg/100 ml in the maternal blood plasma, and 12.2 μg/100 ml in the cord blood plasma. The red blood cells contained no estriol measurable by the present method.

Evidence for the presence of free estriol in plasma of the human maternal peripheral circulation and of umbilical cord blood has been reported in a previous communication from this laboratory (Touchstone and Greene, 1960). The present paper describes methods used and results obtained in the determination of free and conjugated estriol in blood. Estriol is present in the cord blood in the free form and as conjugates of both sulfuric and glucuronic acids. The sulfate conjugate accounted for 77% of the total estriol. The maternal peripheral blood contains free estriol, estriol sulfate, and estriol glucosiduronate. In addition, maternal peripheral blood contains a diconjugated estriol (a 3-sulfate-16- and/or 17-glucosiduronate), which accounted for 44% of the conjugates.

EXPERIMENTAL PROCEDURE

Extraction of Free Estriol.—Cord blood was obtained by milking the umbilical cord at the time of detachment from the fetus. Maternal blood was procured from the antecubital vein during labor or during the last trimester of pregnancy. In order to keep results within the limits of accuracy of the method, aliquots of 10 ml or more of cord blood were extracted in each experiment while 50 ml or more of maternal blood plasma were used.

* This work was supported by Grants ASF-14,013 and A-1509 from the National Institutes of Health and by the John G. Clark Memorial Fund.

The heparinized blood was centrifuged within 15 minutes after withdrawal, and plasma was separated. The plasma was extracted three times with equal volumes of ether (the plasma, after separation, was kept for hydrolysis studies) over periods of 10 minutes each, with vigorous shaking. The combined ether extracts were washed twice with $^1/_{10}$ volumes of 4% bicarbonate, then extracted three times with $^1/_6$ volumes of 1 N sodium hydroxide, and the ether was discarded. The alkali phase was neutralized with 5 N HCl to *p*H 7.0 and extracted three times with equal volumes of ether. The ether was washed once with $^1/_{10}$ volume of 4% $NaHCO_3$ and twice with water, and was evaporated to dryness. The residue so obtained was subjected to column chromatography for separation of free estriol.

Treatment of the Red Blood Cells.—The cells after separation of plasma were washed twice with isotonic saline using a volume equivalent to the original volume of the plasma. Water was then added to hemolyze the cells. The solution so obtained was precipitated with acetone in the same manner as was the plasma, as described later, and estriol extraction was carried out as described previously.

Hydrolysis of Conjugates.—*In acid hydrolysis*, the residual ether present in the plasma extracted was evaporated *in vacuo* to prevent foaming during hydrolysis. The plasma was refluxed for one hour after addition of concentrated HCl to a concentration of 15% by volume. The precipitate was separated by centri-