Quantitative Studies on the Metabolism of Dehydroisoandrosterone Sulfate*

EUGENE SANDBERG,† ERLIO GURPIDE,‡ AND SEYMOUR LIEBERMAN

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

Received March 30, 1964

A mixture of ^{3}H- and ^{35}S-labeled tracers of dehydroisoandrosterone sulfate (DS) and ^{14}C-labeled dehydroisoandrosterone (D) was injected into a normal woman. Following the injection, samples of blood were taken at various intervals for 2 days and the ^{3}H, ^{35}S, and ^{14}C concentrations in DS in the plasma samples were determined. The concentrations of unlabeled DS in plasma were also determined. On the basis of a two-pool (D and DS) model and using equations presented in the preceding paper, values for the volume of distribution of DS, the metabolic clearance rate of DS in plasma, the production rate of plasma DS, the rate of conversion of DS to D, and the rate of excretion and conversion of DS to other products were estimated. Urine was collected for 3 days after the injection and the specific activities of DS and dehydroisoandrosterone glucuronide (DG) isolated from the urine were determined. From these specific activities the rates of secretion and metabolism of DS and of D were estimated. Where comparisons were possible, it was found that the values of the parameters related to the metabolism of DS obtained from plasma data did not significantly differ from those obtained from urinary data. In another experiment the same mixture of tracers was injected into a normal male and blood samples were taken at short intervals during 2 hours following the injection. In this case, the existence of another sulfate pool exchanging with DS in plasma was detected. Its influence on the interpretation of the results obtained from the female subject is discussed.

That dehydroisoandrosterone sulfate (DS)[1] may have a greater physiological importance than that of a mere urinary metabolite is suggested by several recent findings. For example DS, the most abundant steroid in plasma, has been shown to be produced by adrenal tissue both *in vivo* (Baulieu, 1962; VandeWiele *et al.*, 1963) and under *in vitro* conditions (Wallace and Lieberman, 1963; Cohn *et al.*, 1963; Migeon, 1963). In fact, as far as is known, the secretion of DS by the adrenal is unique in that no other product of an endocrine gland is known to be secreted in a conjugated form. Also characteristic is the fact that DS and free dehydroisoandrosterone (D) are interconvertible in normal circumstances (Roberts *et al.*, 1961). Human placental tissue has been shown (Warren and Timberlake, 1962) to convert DS to D, and more surprisingly, the studies of Siiteri and MacDonald (1963) and of Baulieu and Dray (1963) have shown that, in pregnant women, circulating DS is efficiently converted to estrogens. Moreover, the implication that steroid sulfates may have a function other than an excretory one received added support from the work of Calvin (Calvin *et al.*, 1963; Calvin and Lieberman, 1964) who have shown that such conjugates undergo biosynthetic reactions similar to those involved in the formation of steroids customarily considered to be hormones. These facts emphasize the desirability of obtaining additional quantitative information about such conjugates, particularly DS.

* This work was supported in part by U. S. Public Health Service grants (AM-00110 and TI-HD-13-03) and the General Research Support Grant of the National Institutes of Health, U. S. Public Health Service.

† Present address: Department of Obstetrics and Gynecology, Stanford University, School of Medicine, Palo Alto, Calif.

‡ Career Scientist, Health Research Council, City of New York.

[1] Abbreviations used in this paper: D, dehydroisoandrosterone = 3β-hydroxyandrost-5-en-17-one; DG, dehydroisoandrosterone glucuronide = 17-oxoandrost-5-en-3β-yl-β-D-glucopyranosiduronic acid; DS, dehydroisoandrosterone sulfate = 17-oxoandrost-5-en-3β-yl-sulfate; isoandrosterone = 3β-hydroxyandrostan-17-one; ADL, androstenediol = androst-5-ene-3β,17β-diol; ADLS, androstenediol sulfate = 17β-hydroxyandrost-5-en-3β-yl sulfate; XS = unknown steroid sulfate.

In previous studies (VandeWiele *et al.*, 1963) the rates of secretion, production and metabolism of DS have been estimated by the analysis of *urinary* metabolites isolated following the intravenous injection of radioactive steroids. In this paper, the temporal changes of the isotope content of DS in *plasma* have been measured following the intravenous injection of three radioactive tracers. One of the objectives of these experiments was to obtain information about the volume of distribution and the amount of DS in the body as well as values for the rates of metabolism of this conjugate either to the free steroid or to other products. Another aim was to compare the values of the rates of metabolism calculated from the *plasma* data with the values for similar rates obtained by the analysis of *urinary* metabolites. A significant difference in these two sets of values would indicate that the model upon which both calculations were founded is inadequate to represent the situation actually existing in the body. If the two sets of values were found to be similar, then the model in Figure 1 would be consistent with the kinetic behavior of the tracers.

The commonly used *kinetic* method for determining rates of metabolism involves the intravenous injection of a single tracer of the steroid under consideration and the subsequent determination of its disappearance from plasma. This technique is not applicable in the case of DS since this conjugate is interconvertible with D, a substance which is itself secreted and which is metabolized to products other than DS. Thus, neither the analysis of a model consisting of a single pool (Peterson and Wyngaarden, 1956) nor the analysis of a model of two pools which allows for irreversible metabolism from only one of the pools (Tait *et al.*, 1961) is applicable for experiments involving DS.

The simplest descriptive model for a kinetic evaluation would embody secretion into each of two interchanging pools with irreversible metabolism occurring from each. Such a model is depicted in Figure 1. Using experimental designs based on it (cases 2 and 3 in the accompanying paper, Gurpide *et al.*, 1964), it is possible to estimate several kinetic parameters when more than one tracer is administered. Thus, by analyzing the curves of disappearance of the radioactivity associated with DS in plasma after the simultaneous

administration of [^{35}S]DS, [^{3}H]DS and [^{14}C]D, the rate constant of the conversion DS → D, and the rate constant of metabolism of DS by irreversible pathways can be determined. An important technical advantage of such an experiment is that the above parameters can be estimated merely by determining the radioactivity in DS in plasma when its decline with respect to time is linear on a semilogarithmic plot (the "tail" portion of the disappearance curves).

In one of the experiments reported here, a mixture of the three tracers mentioned was injected into a normal female subject. For 2 days after the injection several blood samples were taken and the concentrations of these isotopes in plasma DS were determined. Following the injection of the tracers urine was also collected and the specific activities of urinary dehydroisoandrosterone glucuronide (DG) and of urinary DS were determined. On the basis of the two-pool model, the rates of secretion and metabolism of DS were calculated from these data (Gurpide *et al.*, 1963, 1964).

In another experiment, the same mixture of tracers was injected into a normal male subject and blood samples were taken at short intervals following the injection. In this experiment another sulfate pool exchanging with DS in plasma was detected.

Experimental

Counting Techniques

All assays of radioactivity were performed in a liquid scintillation counter (Tri-Carb Model 314-DS, Packard Instrument Co.). Radioactivity of samples was counted in glass vials (Wheaton Glass Co.) using 5 ml of a toluene solution containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazolyl)-benzene (hereafter referred to as "toluene-phosphor" solution). When assaying samples containing DS, 1 ml of absolute ethanol was added to the vials to increase the solubility of the conjugate (hereafter referred to as "toluene-ethanol-phosphor" solution). Counting was carried out for sufficient time to afford a standard error of the mean of less than 3%.

The samples of D containing ^{3}H and ^{14}C were assayed in toluene-phosphor solution. The contents of the two isotopes in these samples, corrected for quenching, were calculated after the addition of internal standards.[2] These values were further corrected for efficiency under the counting conditions so as to express the results in dpm. The samples of DS containing ^{3}H, ^{14}C, and ^{35}S were assayed in toluene-ethanol-phosphor solution. The extent of quenching owing to colored impurities was determined by comparing the number of cpm produced by known amounts of tracers containing ^{3}H and ^{14}C which were added to the samples and to a toluene-ethanol-phosphor solution.[2] The corrected values, in dpm, of ^{3}H and ^{14}C + ^{35}S were then calculated. ^{14}C and ^{35}S may be considered together since, due to the similarity of the energies of their β emission, the counting efficiencies and the discriminator ratios for each isotope are not significantly different. In order to determine the individual contributions of ^{14}C and ^{35}S to their combined total, the corrected cpm of ^{3}H in DS was divided by the ^{3}H/^{14}C ratio obtained following solvolysis. The quotient represented the cpm of ^{14}C in the combined count of ^{14}C plus ^{35}S in DS. The cpm due to ^{35}S was calculated by subtraction and, after correcting this figure for the radioactive decay of the isotope, the ^{3}H/^{35}S ratio was determined.

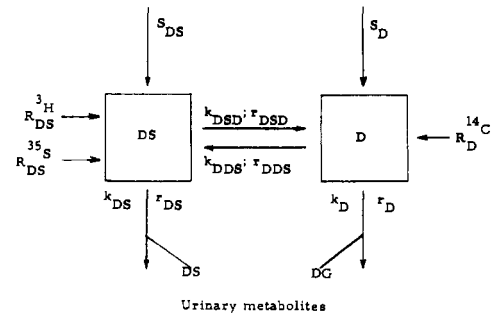

FIG. 1.—Two-pool model assumed to represent the metabolism and interconversion of D and DS. Rates and rate constants are denoted by the r's and the k's, respectively. S_D and S_{DS} are considered to represent the rates of secretion of D and DS. The doses of tracers injected are indicated by the R's.

Chromatography

Paper chromatography was performed on washed Whatman No. 1 paper at room temperature in equilibrated systems. Standards and paper blanks were run on parallel strips in every instance. Column-partition chromatography using Celite as support was carried out as previously described (Gurpide *et al.*, 1962). The solvent systems used in this study are listed in Table I.

Radioactive zones on paper were localized with an automatic paper-chromatogram scanner (Model 880, Vanguard Instrument Co.). 17-Ketosteroids and steroid sulfates were localized on paper by applying the Zimmermann (1936) and the Crepy (methylene blue) (Crepy, 1960) reagents, respectively. Quantitation of D was performed by the micro-Zimmermann technique. Blank values were obtained from eluates of the paper alone.

Preparation and Purification of Tracers

Ammonium Dehydroisoandrosterone-7α-[^{3}H]sulfate.—Dehydroisoandrosterone-7α-[^{3}H]acetate (specific activity 11.6 mc/mg) (New England Nuclear Corp.) was saponified by refluxing for 2 hours in a 2.5% solution of KOH in 50% EtOH. The reaction product was extracted with ethyl acetate and chromatographed in system I, where it was eluted in the sixth HBV (hold-back volume, see Gurpide *et al.*, 1962). The single peak of radioactivity was rechromatographed in system II. The radioactive eluate, which had the same mobility as standard D, was treated with a mixture of 1 ml pyridine and 0.14 ml chlorosulfonic acid. After standing at room temperature overnight, the reaction mixture was treated with 2 ml of water and neutralized with concentrated ammonia. The solution was then extracted with 2 volumes of 1-butanol. The organic extract was evaporated to dryness, under reduced pressure. The residue was chromatographed in system IX (eluted in third HBV) and the radioactive fractions were combined and rechromatographed using system VII.

[2] Quenching in a sample containing ^{3}H and ^{14}C was corrected for in the following manner: (1) The radioactivity of sample was counted to obtain accurate values, N_1 and N_2, in each of the discriminator settings. (2) ^{3}H standard was added to the sample and new values, N_1' and N_2', were obtained. (3) The same amount of ^{3}H added to the sample was counted in toluene-phosphor solution to obtain a value, $N_{1,H}$. (4) and (5) Steps (2) and (3) were repeated using the ^{14}C standard. The corresponding values are N_1'', N_2'', and $N_{2,C}$. (6) The cpm of ^{3}H and ^{14}C in the sample were calculated by applying the formula (Okita *et al.*, 1957): cpm ^{3}H in sample = $b(N_1 - N_2)/(b - a)$ and cpm ^{14}C in sample = $b(N_2 - aN_1)/(b - a)$, where $a = (N_2' - N_2)/(N_1' - N_1)$ and $b = (N_2'' - N_2')/(N_1'' - N_1')$. (7) The values obtained in (6) were corrected for quenching by multiplying each of them by the corresponding factors: $f_{^3H} = N_{1,H}/(N_1' - N_1)$, $f_{^{14}C} = N_{2,C}/(N_2'' - N_2')$.

TABLE I

CHROMATOGRAPHIC SYSTEMS

System	Support	Running Time (hours)	Components	Reference
I	Celite		Isooctane–*t*-butyl alcohol–methanol–water (500:100:350:50)	Siiteri *et al.*, 1963
II	Paper	16	Cyclohexane-dioxane-methanol–water (100:25:100:10)	Siiteri *et al.*, 1963
III	Celite		Isooctane–ethyl acetate–methanol–water (400:130:400:100)	Siiteri *et al.*, 1963
IV	Celite		Isooctane–*t*-butyl alcohol–methanol–water (200:80:80:40)	Siiteri *et al.*, 1963
V	Celite		Isooctane–*t*-butyl alcohol–1 M NH_4OH (1:2:2)	Siiteri *et al.*, 1963
VI	Paper	14	Skellysolve C–90% methanol	Bush, 1952
VII	Celite		Isooctane–*t*-butyl alcohol–1 M NH_4OH (3:5:5)	Siiteri *et al.*, 1963
VIII	Paper	38	Isopropyl ether–Skellysolve B–*t*-butyl alcohol–1.7 M NH_4OH (5:2:3:10)	Baulieu, 1962
IX	Celite		Isooctane–*t*-butyl alcohol–M NH_4OH (2:5:5)	Siiteri *et al.*, 1963
X	Paper	30	Isooctane–ethyl acetate–1-butanol–methanol–1 M NH_4OH (2:4:0.5:2:3)	J. Cos, E. Gurpide, and S. Lieberman (in preparation)
XI	Neutral alumina (6% H_2O)		Gradient elution, benzene–4% ethanol in benzene	Lakshmanan and Lieberman, 1954

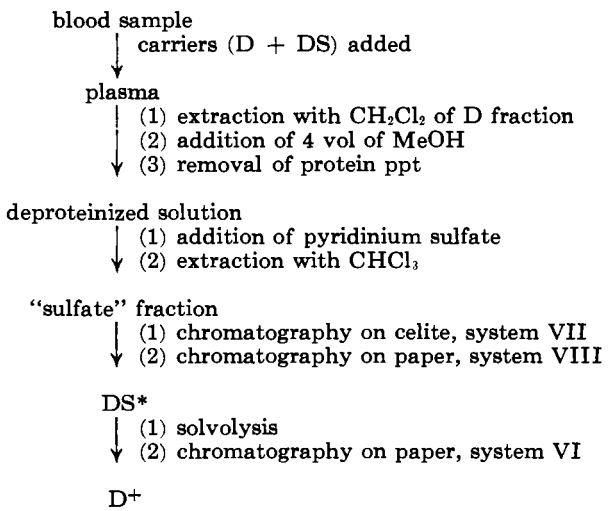

FIG. 2.—Flow sheet outlining procedures used for the estimation of the concentration of ^3H, ^{14}C, and ^{35}S in plasma dehydroisoandrosterone sulfate. * From this sample $^3H/^{14}C + ^{35}S$ ratio (*A*) was determined. + From this sample $\bar{a}_D{}^{3H}$ was estimated from the D content (by Zimmermann assay) and the ^3H content. Also from this sample, $^3H/^{14}C$ ratio (*B*) was determined. From *A* and *B*, $^3H/^{35}S$ ratio (*C*) was calculated. From *B* and $\bar{a}_D{}^{3H}$, $\bar{a}_D{}^{14C}$ was calculated. From $\bar{a}_D{}^{3H}$ and *C*, $\bar{a}_D{}^{35S}$ was calculated.

Ammonium Dehydroisoandrosterone [^{35}S]*Sulfate.*—To 0.7 ml of dry pyridine, cooled in an ice bath, was added 0.05 ml of ^{35}S-labeled chlorosulfonic acid (New England Nuclear Corp.). A solution containing 333 mg of D in 0.2 ml of dry pyridine was then added. A clear solution was obtained by gentle heating. After standing at room temperature for 2 hours, the reaction mixture was diluted with water, made *p*H 8 with concentrated ammonia, and then extracted with 2 volumes of 1-butanol. The organic extract was evaporated to dryness *in vacuo* and the residue was purified by chromatography in system VII. Fractions (eluted in the sixth HBV) containing the lone peak of radioactivity were combined. The solvent was removed and the crystalline residue was recrystallized from methanol. The product had a specific activity of 1.45 μc/mg.

[*4*-^{14}C]*Dehydroisoandrosterone.*—[4-^{14}C]Dehydroisoandrosterone acetate (specific activity 15.2 μc/mg) (New England Nuclear Corp.) was saponified as before. Water was added to the reaction mixture and the product was extracted with ether. The ethereal phase was washed with water and the residue left after evaporation of the solvent was chromatographed using system VI. The solitary fraction of radioactivity was eluted and was rechromatographed using system I from which it was eluted in the sixth HBV.

Radiochemical Homogeneity of Tracers.—Proof of radiochemical homogeneity of the tracers is given in Table II in which the constancy of the specific activities of the products obtained by crystallization with authen-

TABLE II

TESTS FOR RADIOCHEMICAL HOMOGENEITY OF THE TRACERS

	Tracers Added to Carriers	Specific Activities (dpm/μmole) Isotope	Crystals	Mother Liquor
I	[^{14}C]D	^{14}C	4,800	5,100
II	[^{35}S]DS	^{35}S	13,400	13,500
III	[^3H]DS + [^{35}S]DS	^3H	114,000	106,000
		^{35}S	21,100	20,600
IV	Same as (III) reduced with $NaBH_4$	^3H	104,000	
		^{35}S	19,700	
V	[^3H]D derived from [^3H]DS (calculated specific activity = 112,000)	^3H	118,000	113,000
VI	[^3H]D from (V) + [^{14}C]D	^3H	17,500	17,700
		^{14}C	20,300	19,500
VII	Same as (VI) reduced with $NaBH_4$, chromatographed, and recrystallized	^3H	17,500	
		^{14}C	18,500	

tic carriers and by formation of derivatives is shown. 7α-[^{3}H]DS and [^{35}S]DS were rechromatographed immediately before injection to remove any products of spontaneous hydrolysis. In neither case was extraneous radioactivity found.

In Vivo Experiments Using Three Tracers

A. Female Subject.—The subject was a normal 35-year-old female weighing 49 kg. A blood sample was taken at noon for determination of the hematocrit and the plasma concentration of DS. Immediately thereafter, [4-^{14}C]D (1.72×10^6 dpm), 7α-[^{3}H]DS (40.5×10^6 dpm), and [^{35}S]DS (2.93×10^6 dpm) were simultaneously injected into an antecubital vein in 7% ethanol. Heparinized blood samples, measuring approximately 100 ml each, were then collected 2, 10, 24, 34, and 48 hours after the injection.

To allow subsequent correction for losses incurred during extraction and purification, 4.56×10^6 dpm 7α-[^{3}H]DS was added to the preinjection blood sample in 1 ml of absolute ethanol. To each postinjection blood sample 1.99 mg (6.91 μmole)[3] of unlabeled D and 2.00 mg (5.19 μmoles) of DS dissolved in 1 ml of propylene glycol were added. Following the addition, each blood sample was equilibrated at room temperature for 1 hour. After its volume was determined, the plasma from each sample was separated by centrifugation. The red cells were washed twice with equal volumes of saline. The plasma and saline washes were combined and frozen.

A 3-day urine sample, 2000 ml, was collected and frozen.

ANALYSIS OF POST-INJECTION BLOOD SAMPLES (Fig. 2).—The combined plasma and saline washes of each postinjection blood sample were thawed and the unconjugated steroids were removed by extraction first with dichloromethane and then with ethyl ether. The treatment of these organic extracts is described below. Four volumes of absolute methanol were added to the aqueous phase and the resulting mixture was refrigerated overnight at 4°. The precipitate which had formed was removed by centrifugation and was washed twice with 80% methanol. The methanol was stripped from the combined supernatant and washes and the resulting aqueous phase was extracted with hexane to remove fat. The steroidal sulfates were then extracted from the water with chloroform after the addition of enough pyridinium sulfate (McKenna and Norymberski, 1960) to make the final concentration 0.3 M. Evaporation of the solvent left a residue which was further purified by chromatography on Celite using system VII. In this system, the monosulfates of the common C-19 steroids were eluted in 4–6 HBV's. Chromatography on paper in system VIII separated DS from other monosulfates. Of the labeled steroid conjugates likely to be encountered in this study, only DS and androst-5-ene-3β,17β-diol-3-sulfate are known to have the same chromatographic mobility in this system. Reference standards were always run on parallel strips in order to corroborate the separation of DS and isoandrosterone sulfate whose polarities in this system differ only slightly.

After localization and elution of DS, two aliquots were removed for the determination of the ^{3}H/^{14}C + ^{35}S ratio. The remainder of the sample was solvolyzed with tetrahydrofuran containing $HClO_4$ (Burstein and Lieberman, 1958) and the liberated steroid was chromatographed on paper using system VI wherein D and androst-5-ene-3β,17β-diol are separable. The location of D was determined by scanning for radioactivity and by the Zimmermann reaction. No radioactivity corresponding to the mobility of the diol was noted in any of the plasma samples. D was eluted and its specific activities with respect to ^{3}H and ^{14}C were determined. The total weight of D was determined by the Zimmermann reaction and from this it could be estimated that the recovery of the DS added as carrier was 25%.

The specific activity of DS with respect to ^{35}S was determined by proportionality using the specific activity with respect to ^{3}H of D and the ^{3}H/^{35}S ratio obtained in the aliquot of DS counted before solvolysis (see under Counting Techniques). The quantity of each of the three isotopes present in DS per 100 ml of plasma at the time of each sampling was then expressed as the percentage of the total amount of that isotope injected.

The dichloromethane and ether extracts of each blood sample were combined, washed with 0.1 N NaOH and with water. After removal of the solvents, the residue was chromatographed on paper using system VI. Samples of unconjugated D (originating from the radioactive plasma samples and the carrier) were localized and eluted with methanol. Their specific activities were determined for both ^{3}H and ^{14}C. In samples of blood removed 2, 10, and 24 hours following the injection of radioactivity, the percentage of the injected ^{3}H present in D in 100 ml of plasma was, respectively, 0.006, 0.012, and 0.003. Of the injected ^{14}C, 0.38% was present in D per 100 ml of plasma in the first sample after injection. In all other instances the concentration of isotopes in plasma D was too low to be determined, and as a result it was not possible to calculate the various kinetic parameters that characterize the D-plasma pool. The average recovery of carrier D was 40%.

DETERMINATION OF THE PLASMA CONCENTRATION OF DS IN THE PREINJECTION SAMPLE.—Tritiated DS was added to the blood sample obtained before the injection of the tracers. The sample was then processed in the manner described. The specific activity was determined on samples of D recovered after hydrolysis of the isolated DS. No radioactivity was detected in the methylene chloride and ether extracts of this blood sample, indicating that hydrolysis of the added isotopically labeled tracer had not occurred during the hour-long equilibration at room temperature.

ANALYSIS OF THE URINARY METABOLITES.—Ninety per cent of the defrosted urine was used for the determination of the cumulative specific activities with respect to ^{3}H and ^{14}C of DG and DS. This portion of the pooled urine was extracted according to the method of Edwards *et al.* (1953). The resulting extract was chromatographed using system IX in which the steroid monosulfates (2–4 HBV) are separated as a group from the glucuronoside conjugates (4–6 HBV).

The *sulfate* fraction was rechromatographed on system VII and then the purified material was further fractionated on paper using system VIII. A single zone of radioactivity having the mobility common to both DS and androst-5-ene-3β,17β-diol monosulfate was recovered from the paper. Carrier DS was added to an aliquot of this fraction and the mixture was chromatographed using system X in which DS and the diol monosulfate are separable. Only a single peak of radioactivity corresponding to the carrier DS was detected. This material was eluted from the paper with methanol and the ^{3}H/^{14}C + ^{35}S ratio of the residue was determined. The remainder of the single peak of radioactivity separated in system VIII was solvolyzed

[3] Molecular weight of D = 288.4. Molecular weight of DS (as D-O-SO_3^-) = 367.4.

and the resulting steroid was chromatographed in system VI. The single peak of radioactivity, showing the same mobility as standard D, was eluted and its cumulative specific activity with respect to both ^{3}H and ^{14}C was determined. The specific activity with respect to ^{35}S of the urinary DS was calculated using the $^{3}H/^{35}S$ ratio as described above for the blood samples.

The *glucuronoside* conjugates, isolated as a group in system IX (*vide supra*) were dissolved in water which was then brought to *p*H 5.0 with sodium acetate buffer. After the addition of 900,000 units of β-glucuronidase ("Ketodase," Warner-Chilcott Lab.) the mixture was incubated at 37° for 84 hours. The free steroids were extracted into ethyl acetate after which the organic extract was washed with a 5% solution of NaOH and then with water. The extract was dried over sodium sulfate, following which the solvent was evaporated under reduced pressure. The residue was chromatographed in system XI and the radioactive peak containing D and possibly isoandrosterone (derived from their glucuronosides) was further chromatographed using system VI, which can resolve these compounds. One radioactive region, having the mobility of standard D, was detected. The central portion was eluted with methanol and the eluate was rechromatographed in the same system to ensure homogeneity, which was judged by the symmetry of the radioactive peak. The cumulative specific activities with respect to both ^{3}H and ^{14}C for the D purified in this way were then determined.

Due to the low specific activity of the injected [^{35}S]DS, 904 μg of the steroid was injected into the subject. Consequently it was necessary to correct the observed specific activities of urinary DS and DG for the weight of the injected tracer. The corrected specific activities were calculated in the following manner. To facilitate the calculation, it was postulated that all of the DS introduced with the tracers was associated with the tritium label and none with the ^{14}C and ^{35}S tracers. The exogenously introduced weight present in the metabolites could then be estimated from their tritium content. Since 40.5×10^{6} dpm of ^{3}H and 2.34 μmoles of DS were injected, 5.78×10^{-8} μmoles corresponded to 1 dpm. A number of μmoles, equal to the ^{3}H content (dpm) multiplied by the factor 5.78×10^{-8}, were subtracted from the weight (w) of the sample of the metabolite to obtain the corrected specific activity. Thus

$$\bar{a}^{3}H_{DS,corr} = \frac{\text{dpm } ^{3}H}{w - 5.78 \times 10^{-8} \text{ dpm } ^{3}H}$$

or

$$\bar{a}^{3}H_{DS,corr} = \frac{\text{dpm } ^{3}H/w}{1 - 5.78 \times 10^{-8} \text{ dpm } ^{3}H/w} = \frac{\bar{a}^{3}H_{DS,obs}}{1 - 5.78 \times 10^{-8} a^{3}H_{DS,obs}}$$

and

$$\bar{a}^{3}H_{DG,corr} = \frac{\bar{a}^{3}H_{DG,obs}}{1 - 5.78 \times 10^{-8} a^{3}H_{DG,obs}}$$

The corrected specific activities with respect to ^{14}C and ^{35}S were calculated by proportionality from the experimentally determined $^{3}H/^{14}C$ and $^{3}H/^{35}S$ ratios.

The remaining one-tenth of the pooled urine was used to determine the average daily excretion of DS during the time urine was collected. For the reverse isotope-dilution procedure, 2.00 mg of unlabeled ammonium DS (5.19 μmole) in 2 ml of absolute ethanol was added to this portion of the urine. The steroid sulfate conjugates were selectively extracted with chloroform after diluting the urine to 0.3 M with pyridinium sulfate. The organic solvent was evaporated under vacuum and the residue was solvolyzed in tetrahydrofuran-containing $HClO_4$ (Burstein and Lieberman, 1958). The product of solvolysis was chromatographed using system I, and fractions exhibiting both radioactivity and a positive Zimmermann chromogen were rechromatographed using system VI. The central portion of the major peak of radioactivity, coinciding in mobility with the carrier D, was then rechromatographed in the same system. The single region of radioactivity was eluted with methanol and, after removal of the solvent, the specific activity of the residue with respect to both ^{3}H and ^{14}C was determined.

B. Male Subject.—A 31-year-old normal male was injected with the same mixture of tracers described in the previous experiment. In this case, however, blood samples were taken at short intervals during the first 2 hours after the injection (see Table III). The blood samples were processed by procedures similar to those used for the samples obtained from the female subject.

TABLE III

ISOTOPE CONCENTRATION IN DS IN PLASMA OF A 31-YEAR-OLD NORMAL MALE SUBJECT AFTER ADMINISTRATION OF [^{35}S]DS, [^{3}H]DS, AND [^{14}C]D

Sample	Time after Injection (minutes)	Per Cent Injected Dose in DS per 100 ml of Plasma		
		^{35}S	^{3}H	^{14}C
A	11	3.36	2.98	0.62
B	18	2.65	2.29	0.73
C	23	2.56	2.26	0.86
D	34	2.13	1.91	0.84
E	53	1.76	1.72	0.80
F	65	1.67	1.60	0.75
G	74	1.59	1.59	0.73
H	94	1.57	1.45	0.73
I	124	1.40	1.25	0.66

RESULTS

A. Female Subject.—Table IV shows the time of collection and volume of the blood samples, the amounts of DS carrier added to each, the final specific activities of DS recovered from these samples with respect to ^{3}H, ^{14}C, and ^{35}S, and the ratios, $^{3}H/^{14}C$ and $^{3}H/^{35}S$ in DS in each. The total amount (dpm) of each isotope in each of the samples is equal to the product of the corresponding specific activities and the weight of the DS added. Using the sample volume and the hematocrit value obtained from the preinjection blood sample (Table V), the isotope concentrations in DS may be expressed as per cent of the injected dose per 100 ml of plasma. These calculated values are shown in the last three columns of Table IV.

In Figure 3 the isotope concentrations in DS in plasma are plotted on a semilogarithmic scale against the time elapsed following the injection of the tracers. It can be seen that, for each of the isotopes, the experimental points may be adequately fitted by a straight line. With respect to ^{3}H and ^{14}C, the straight lines formed by the experimental points (Fig. 3) indicate that the slow component of the exponential curve of disappearance of these isotopes (described by equations 15 and 19 in the accompanying paper) is dominant and that the terms containing the factor $e^{-\alpha t}$ in these equations can be neglected. It should be noted that the first experimental points on the curves for both ^{3}H and ^{14}C belong to the straight lines formed by the suc-

TABLE IV

SUMMARY OF BLOOD-SAMPLE DATA AND RESULTS FROM FEMALE SUBJECT OBTAINED FOLLOWING INJECTION OF TRACERS[a]

Time of Collection (hours after injection)	Volume of Blood Sample (ml)	Carrier Added (μmoles)		Specific Activity (in dpm/μmole) of DS in Plasma with Respect to:					Per Cent Injected Dose in DS per 100 ml of Plasma		
		D	DS	^{3}H	^{14}C	^{35}S	$^{3}H/^{14}C$	$^{3}H/^{35}S$	^{3}H	^{14}C	^{35}S
2	75	6.91	5.19	74,000	1600	4800	46.4	15.3	2.14	1.09	1.93
10	90	6.91	5.19	41,000	870	2000	46.9	20.4	0.99	0.49	0.67
24	130	6.91	5.19	24,000	530	790	45.6	30.6	0.40	0.21	0.18
34	108	6.91	5.19	8,900	180	225	49.2	40.0	0.18	0.09	0.06
48	112	6.91	5.19	4,100	96	57	43.2	72.8	0.08	0.04	0.01

[a] Samples collected at intervals following the simultaneous intravenous injection of [^{35}S]DS, [^{3}H]DS, and [^{14}D]D ($^{3}H/^{14}C$ injected, 23.5).

TABLE V

SUMMARY OF BLOOD-SAMPLE DATA AND RESULTS FROM FEMALE SUBJECT OBTAINED IMMEDIATELY PRIOR TO INJECTION OF TRACERS

Volume of Blood Sample (ml)	Amount of [^{3}H]DS Added to Sample (dpm)	Specific Activity of Isolated DS (dpm/μmole)	Hematocrit (%)	Concentration of Endogenous DS (μmole per 100 ml)	
				Blood	Plasma
108	4.56×10^{3}	19.9×10^{5}	41	0.21	0.36

TABLE VI

ISOTOPE CONTENT IN URINARY METABOLITES[a]

Conjugate	$\bar{a}^{3}H$ [b]	$\bar{a}^{14}C$ [b]	$\bar{a}^{35}S$	$^{3}H/^{14}C$
DS	720,000	14,700	33,500	48.9
DG	530,000	36,000		14.7

[a] Isotope contents of DS and DG recovered from 0.9 of the 3-day urine specimen from female subject. [b] Calculated from the corresponding isotope contents of dehydroisoandrosterone derived by cleavage of the conjugates, dehydroisoandrosterone sulfate, and dehydroisoandrosterone glucuronoside. The specific activities are expressed in dpm/μmole.

ceeding points. This follows from the fact that all samples possess the same $^{3}H/^{14}C$ ratio (Table IV) and this may be taken as proof that all the points lie on the "tail" of the disappearance curve (Gurpide *et al.*, 1964). Thus it is justifiable to use all the experimental points in the construction of the straight line representing the disappearance of ^{3}H and ^{14}C from plasma DS.

Table V shows the data used to estimate the concentration of DS in blood and in plasma. The concentration of DS in peripheral blood was calculated by dividing the number of dpm added by the product of the specific activity of the isolated DS and the volume of the sample. The corresponding plasma concentration was calculated on the basis of the hematocrit value.

Table VI presents the specific activities with respect to ^{3}H and ^{14}C of D recovered from its sulfate (DS) and D recovered from its glucuronoside (DG), isolated from a sample of urine collected for 3 days following the injection of the tracers. These specific activities have been corrected to account for the weight of DS introduced with the tracers. Also indicated in Table VI is the specific activity with respect to ^{35}S of DS and the ratios of $^{3}H/^{14}C$ found in these urinary conjugates.

The daily urinary excretion of DS (ϵ_{DS}) was estimated by a reverse isotope-dilution procedure and the result is given in Table VII. The analysis was carried out on one-tenth of the pooled 3-day urine specimen. In the table, W is the amount of DS carrier added and a

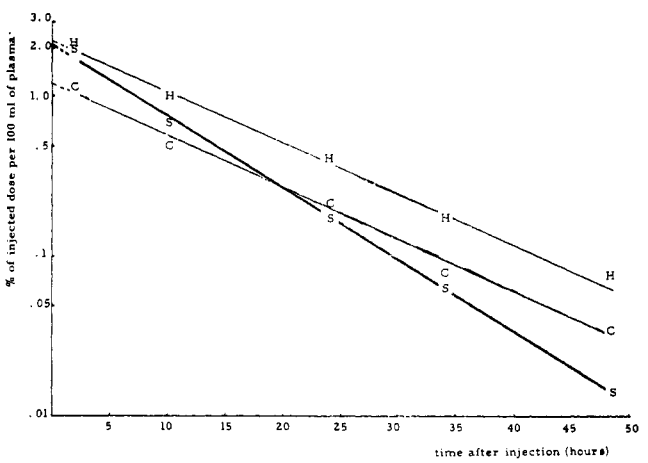

FIG. 3.—Semilogarithmic plot of the percentages of the doses of isotopes injected present in DS in 100 ml of plasma in the female subject against the time of sampling after the simultaneous injection of [^{35}S]DS, [^{3}H]DS, and [^{14}C]D. The straight lines were calculated by using the method of least squares. H, C, and S represent the experimental points corresponding to ^{3}H, ^{14}C, and ^{35}S, respectively.

TABLE VII

DATA USED TO CALCULATE DAILY EXCRETION OF DEHYDROISOANDROSTERONE SULFATE IN THE FEMALE SUBJECT

Amount (W) of Carrier DS Added to 1/10 Urine Volume (μmole)	Specific Activity ($a_{DS}{}^{3}H$) of DS isolated from 1/10 Urine Volume (dpm/μmole)	$\bar{a}_{DS}{}^{3}H$ in 9/10 Urine Volume (dpm/μmole) (from Table VI)	Daily Excretion of DS(ϵ_{DS}) (μmole)
5.19	6000	720,000	0.14

is the specific activity with respect to ^{3}H of DS isolated from the sample. $\bar{a}_{DS}{}^{3}H$ symbolizes, as in Table VI, the cumulative specific activity with respect to ^{3}H of DS isolated from the other nine-tenths of the pooled urine to which no carrier was added. Since the number of dpm

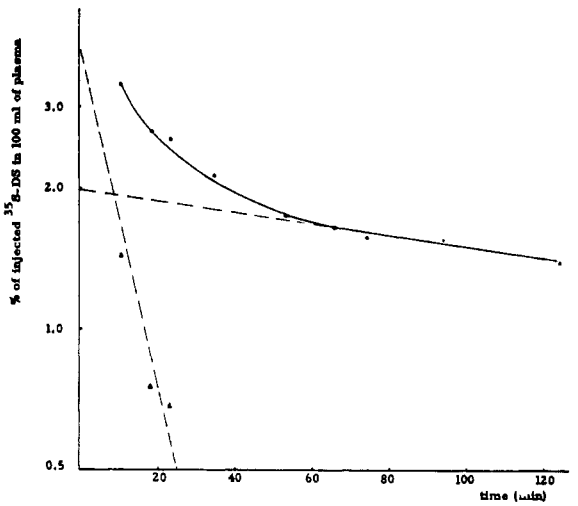

FIG. 4.—Semilogarithmic plot of the percentage of injected [^{35}S]DS present in 100 ml of plasma in the male subject at intervals following the injection of the tracer. ●, experimental points; △, differences between the experimental points and the extrapolated line (see text for explanations). The values corresponding to the 34- and 53-minute samples are not shown. The straight lines were calculated by using the method of least squares.

of ^{3}H present in DS in the total urine volume is ten times the number present in the aliquot, the following equation results:

$$10a\left(\frac{3\epsilon_{DS}}{10} + w\right) = 3\epsilon_{DS}\bar{a}^{3}H_{DS}$$

Then

$$\epsilon_{DS} = \frac{10aw}{3(\bar{a}^{3}H_{DS} - a)}$$

B. Male Subject.—Table III shows the percentages of the injected doses of each isotope present in DS in 100 ml of plasma determined in each of the samples. The concentrations corresponding to the ^{35}S isotope are presented in a semilogarithmic plot against time in Figure 4. It can be seen from Table III that the ^{3}H curve, if drawn, would be practically superimposed upon the ^{35}S curve in Figure 4, and that the maximum concentration of ^{14}C in DS was reached in the sample taken at 23 minutes after the injection. The concentration of DS was estimated as 0.73 μmole per 100 ml of plasma.

CALCULATIONS

A. *From Results Obtained from Female Subject*

1. Calculations from Data Obtained from Blood Samples.—(A) RATE CONSTANT OF TOTAL REMOVAL ($k_{DS} + k_{DSD}$) AND RATE OF TOTAL REMOVAL ($r_{DS} + r_{DSD}$) FROM THE DS POOL, POOL SIZE (M_{DS}) OF DS AND VOLUME OF DISTRIBUTION (V_{DS}) OF DS.—Using the model in Figure 1 to represent the naturally occurring situation, the equation describing the straight line obtained when the per cent of the administered dose of ^{35}S found in DS per 100 ml of plasma is plotted on a semilogarithmic scale against the time of sampling is given by (see accompanying paper):

$$\log\frac{10y^{35}S_{DS}}{R^{35}S_{DS}V_{DS}} = -0.4343(k_{DS} + k_{DSD})t + \log\frac{10}{V_{DS}}$$

where V_{DS} is the volume of distribution of DS (in liters) assuming that the plasma concentration of DS is equal to its concentration in the total space, $y_{DS}{}^{35}S$ is the quantity (dpm) of ^{35}S in the DS pool, and $R_{DS}{}^{35}S$ is the quantity (dpm) of ^{35}S injected as DS. From the values for the slope and the intercept with the ordinate axis (Fig. 3), obtained analytically by use of the least squares equation, the following values are obtained:

$$k_{DS} + k_{DSD} = 0.103\ hr^{-1}$$

$$V_{DS} = 4.7\ liters$$

The half-life of the ^{35}S in the DS pool therefore is

$$t_{1/2} = \frac{0.693}{k_{DS} + k_{DSD}} = 6.7\ hours$$

The total amount of DS in the pool (M_{DS}) is obtained as the product of the volume of distribution and the plasma concentration (c_{DS}) of DS which was determined in a sample taken prior to the injection of the tracers (Table V) and was assumed to remain constant throughout the experiment. Thus,

$$M_{DS} = V_{DS}c_{DS} = 16.8\ \mu moles$$

Here M_{DS} is used to calculate the rate of total removal of DS from the pool in the following manner:

$$r_{DS} + r_{DSD} = (k_{DS} + k_{DSD})M_{DS} = 1.73\ \mu moles\ hr^{-1}$$

In making the calculations shown, the possibility of the existence of another pool exchanging with plasma DS without loss of ^{35}S has been ignored. However, the existence of such pool would result in an overestimation of V_{DS} by the procedure described. Taking the volume of plasma, V_{pl}, as the lower limit for the volume of distribution of DS introduced into circulation, lower limits for the values of M_{DS} and $r_{DS} + r_{DS}$ can be established assuming that the existence of a second sulfate pool does not affect significantly the calculated values of $k_{DS} + k_{DSD}$. This assumption could not be validated and introduces a degree of uncertainty in the calculated values (see Discussion). If V_{pl} is estimated as 5% of the body weight (Gamble, 1952), then $V_{pl} = 2.4$ liters, $M_{DS} = 8.7$ μmoles, and $r_{DS} + r_{DSD} = 0.90$ μmole hr^{-1}.

(B) RATE CONSTANT (k_{DSD}) AND RATE OF TRANSFER (r_{DSD}) FROM THE DS TO THE D POOL.—As mentioned previously, the straight lines corresponding to ^{3}H and ^{14}C shown in Figure 3 represent the "tail" portion of the disappearance curves in which the slow-component term is dominant. The value of β in these exponential terms (see equations 15 and 19 in the accompanying paper) is calculated from the slopes of these lines. Identical values, $\beta = 0.070\ hr^{-1}$, are obtained from each of the lines by the least squares method. The ratio $^{3}H/^{14}C$ calculated from these two parallels is 47.1.

Equation (23) in the accompanying paper describes a useful relationship by which the rate constant of conversion of DS to D(k_{DSD}) can be calculated from experimental data obtained from the measurement of the ratio $^{3}H/^{14}C$ in DS; and the slopes of the [^{3}H]DS and [^{35}S]DS curves, i.e.,

$$(^{3}H/^{14}C)_{DS,constant} = \frac{R_{DS}{}^{3}H}{R_{D}{}^{14}C}\frac{k_{DSD}}{(k_{DSD} + k_{DS}) - \beta}$$

or

$$k_{DSD} = \frac{(^{3}H/^{14}C)_{DS,constant}}{(^{3}H/^{14}C)_{injected}}[(k_{DSD} + k_{DS}) - \beta]$$

Substituting the values previously calculated,

$$k_{DSD} = \frac{47.1}{23.5}(0.103 - 0.070)\ hr^{-1} = 0.066\ hr^{-1}$$

Then, the conversion factor (Gurpide *et al.*, 1963)

$$\rho_{DSD} = \frac{k_{DSD}}{k_{DS} + k_{DSD}} = 0.63$$

The rate of conversion of DS to D (r_{DSD}) is obtained by multiplying the corresponding rate constant (k_{DSD}) by the amount of DS in the pool (M_{DS}). As discussed previously, the value of M_{DS} has been considered to lie between two limits. The upper limit, obtained by assuming that DS in plasma does not exchange with another sulfate pool, is 16.8 μmoles, from which r_{DSD} = 1.10 μmoles hr^{-1}. The lower limit, obtained assuming that the volume of the DS pool equals the plasma volume, is 8.7 μmoles from which a value of r_{DSD} = 0.57 μmole hr^{-1} is obtained.

The rate constant of removal of material from the DS pool via irreversible pathways (k_{DS}) can be calculated by subtraction as follows:

$$k_{DS} = (k_{DS} + k_{DSD}) - k_{DSD} = 0.037 \text{ hr}^{-1}$$

By considerations similar to those mentioned above, the rate at which this process occurs (r_{DS}) can be estimated to lie between two limits:

$$r_{DS} = k_{DS} \times M_{DS} = 0.037 \times 16.8 = 0.62 \text{ μmole/hour}$$

or

$$r_{DS} = 0.037 \times 8.7 = 0.32 \text{ μmole/hour}$$

(c) Metabolic clearance rate and production rate of DS in plasma (MCR_{DS} and PR_{DS}).—As indicated by Tait (1963) and in the accompanying paper (equation 36), the metabolic clearance rate of DS in plasma can be estimated from the area under the curve resulting from the plot of the concentration of ^{3}H-DS in plasma against time. Since in this experiment data describing the early part of that curve (prior to the 2-hour sample) was not obtained, the contribution of the 0–2-hour region of the curve to the total area is unknown. However, the difference between the area under the total curve and the area under the curve resulting from the extrapolation of the experimental curve to zero time may be negligible considering the slow rate of removal of DS from the pool. Then, the extrapolation of the [^{3}H]DS concentration curve to zero time should be adequate to evaluate MCR_{DS}. For this purpose, therefore, the function of time giving the concentration of [^{3}H]DS in plasma may be expressed as a single exponential term. Consequently, as indicated in equation (37) in the accompanying paper, MCR_{DS} can be estimated from β and the intercept of the ^{3}H line in Figure 3 with the ordinate axis (intercept = 2.2% of the [^{3}H]DS injected per 100 ml of plasma),

$$MCR_{DS} = \frac{10 \times 0.070 \text{ liter}}{2.2} \frac{\text{liter}}{\text{hour}} = 0.32 \frac{\text{liter}}{\text{hour}}$$

The production rate of DS in plasma will then be (Tait, 1963; Gurpide *et al.*, 1964)

$$PR_{DS} = MCR_{DS} \times c_{DS}$$
$$= 0.32 \times 3.6 \text{ μmoles/hour} = 1.15 \text{ μmoles/hour}$$

2. Calculations from Data Obtained from Urinary Metabolites.—(a) Rate of total removal from the DS pool ($r_{DS} + r_{DSD}$).—(i) From the cumulative specific activity of urinary DS with respect to ^{35}S($\bar{a}_{DS}^{35S}$). As previously mentioned, it has been assumed that the tracer [^{35}S]DS does not re-enter the DS pool. Consequently, the dilution of the isotope associated with DS will be due to the re-entry of new DS as well as material which is being recycled. The formula for the calculation of the secretory rate of hormones which is based on models consisting of a single pool (Peterson and Wyngaarden, 1956) can be used to estimate the rate of total entry of material into the pool and the rate of total removal from the pool since these two rates should be identical in the steady state. Thus,

$$r_{DS} + r_{DSD} = S_{DS} + r_{DDS} = \frac{R_{DS}^{35S}}{\bar{a}_{DS}^{35S} \times t}$$

where $\bar{a}_{DS}^{35S}$ is the cumulative specific activity with respect to ^{35}S of DS isolated from urine which has been collected for t days after injection of the tracer. In the present experiment, urine was collected for 3 days, a period of time which has previously been shown (MacDonald *et al.*, 1963) to be sufficiently long for almost total excretion of the radioactivity that appears in urinary DS. Substituting the appropriate numerical data in the previous equation, the following value for the rate of total removal is obtained:

$$r_{DS} + r_{DSD} = \frac{2.93 \times 10^6 \text{ dpm}}{33{,}500 \text{ dpm}/\mu\text{mole} \times 3 \text{ days}}$$
$$= 29.1 \text{ μmoles/day} = 1.21 \text{ μmoles/hr}$$

(ii) From the cumulative specific activities of urinary DS and DG with respect to ^{3}H and ^{14}C. As described further on in detail, r_{DS} and r_{DSD} can be evaluated individually from the cumulative specific activities with respect to ^{3}H and ^{14}C of urinary DS and DG. The sum of these values, $r_{DS} + r_{DSD}$ = 0.41 + 0.69 = 1.10 μmoles/hr, is in close agreement with the above value estimated from the ^{35}S cumulative specific activity of urinary DS.

(b) Rates of secretion (S_{DS},S_D), of interconversion (r_{DSD},r_{DDS}), of metabolism via irreversible pathways (r_{DS},r_D) and of production (PR_{DS}, PR_D) of DS and D.—The determination of these rates from the specific activities of urinary DS and DG with respect to ^{3}H and ^{14}C, following the injection of [^{3}H]DS and ^{14}C-D, has been previously discussed (Gurpide *et al.*, 1963). The symbols and formulas described in that publication are used here unaltered. Substituting the appropriate experimental data from Table VI, the following values for these rates are obtained:

$$S_{DS} = \frac{R_{DS}^{3H}}{t} \cdot \frac{\bar{a}_{DG}^{14C} - \bar{a}_{DS}^{14C}}{\bar{a}_{DG}^{14C}\bar{a}_{DS}^{3H} - \bar{a}_{DS}^{14C}\bar{a}_{DG}^{3H}}$$
$$= 15.78 \text{ μmoles/day} = 0.65 \text{ μmole/hour}$$

$$S_D = \frac{R_D^{14C}}{t} \cdot \frac{\bar{a}_{DS}^{3H} - \bar{a}_{DG}^{3H}}{\bar{a}_{DG}^{14C}\bar{a}_{DS}^{3H} - \bar{a}_{DS}^{14C}\bar{a}_{DG}^{3H}}$$
$$= 6.12 \text{ μmoles/day} = 0.25 \text{ μmole/hour}$$

$$r_{DSD} = \frac{R_D^{14C}}{t} \cdot \frac{\bar{a}_{DG}^{3H}}{\bar{a}_{DG}^{14C}\bar{a}_{DS}^{3H} - \bar{a}_{DS}^{14C}\bar{a}_{DG}^{3H}}$$
$$= 16.67 \text{ μmoles/day} = 0.69 \text{ μmole/hour}$$

$$r_{DDS} = \frac{R_{DS}^{3H}}{t} \cdot \frac{\bar{a}_{DS}^{14C}}{\bar{a}_{DG}^{14C}\bar{a}_{DS}^{3H} - \bar{a}_{DS}^{14C}\bar{a}_{DG}^{3H}}$$
$$= 10.96 \text{ μmoles/day} = 0.45 \text{ μmole/hour}$$

$$r_{DS} = S_{DS} + r_{DDS} - r_{DSD}$$
$$= 10.07 \text{ μmoles/day} = 0.41 \text{ μmole/hour}$$

$$r_D = S_D + r_{DSD} - r_{DDS}$$
$$= 11.83 \text{ μmoles/day} = 0.49 \text{ μmole/hour}$$

$$PR_{DS} = \frac{R_{DS}^{3H}}{\bar{a}_{DS}^{3H} \times t} = 18.73 \text{ μmoles/day} = 0.78 \text{ μmole/hour}$$

$$PR_D = \frac{R_D^{14C}}{\bar{a}_{DG}^{14C} \times t} = 15.95 \text{ μmoles/day} = 0.66 \text{ μmole/hour}$$

The conversion factors ρ_{DSD} and ρ_{DDS} in the expressions $PR_D = S_D + \rho_{DSD}S_{DS}$ and $PR_{DS} = S_{DS} + \rho_{DSS}S_D$ are also obtained from the urinary data. Thus,

$$\rho_{DDS} = \frac{(^3H/^{14}C)_{DG}}{(^3H/^{14}C)_{\text{injected}}} = 0.62$$

$$\rho_{DDS} = \frac{(^3H/^{14}C)_{\text{injected}}}{(^3H/^{14}C)_{DS}} = 0.48$$

(c) Metabolic clearance rate of DS (MCR_{DS}).—As discussed, this parameter may be calculated from

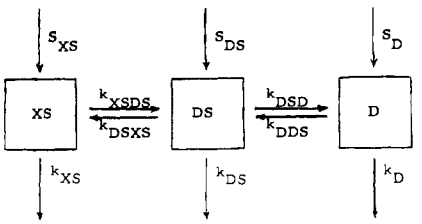

FIG. 5.—Three-pool model in which two sulfate pools (DS and XS) exchange. See text for explanation of symbols.

the production rate and the concentration of DS in plasma. Thus,

$$MCR_{DS} = \frac{PR_{DS}}{c_{DS}} = 5.21 \text{ liters/day} = 0.22 \text{ liter/hour}$$

For comparison, values for parameters calculated from the experimental data derived from the analysis of blood and urine are presented in Table VIII. The values for other parameters are shown in Table IX.

TABLE VIII
COMPARISON OF KINETIC PARAMETERS OF METABOLISM OF DS[a]

Parameter	Plasma		Urine
PR_{DS} (μmole/hr)	1.15		0.78
MCR_{DS} (liter/hr)	0.32		0.22
ρ_{DSD}	0.63		0.62
	Upper Limit[b]	Lower Limit[b]	
$r_{DSD} + r_{DS}$ (μmole/hr)	1.73	0.90	1.21,[c] 1.10[d]
r_{DSD} (μmole/hr)	1.10	0.57	0.69
r_{DS} (μmole/hr)	0.62	0.32	0.41

[a] From data obtained from plasma and urine of female subject. [b] See text. [c] From [^{35}S]DS specific activity. [d] From ^{3}H and ^{14}C data.

TABLE IX
OTHER PARAMETERS RELATING TO DS FROM DATA OBTAINED FROM THE PLASMA OF THE FEMALE SUBJECT

Parameter	Value
c_{DS}	3.6 μmoles/liter
k_{DSD}	0.066 hr^{-1}
k_{DS}	0.037 hr^{-1}
V_{DS}	Upper limit = 4.7 liters
	Lower limit = 2.4 liters
M_{DS}	Upper limit = 16.8 μmoles
	Lower limit = 8.7 μmoles

B. From Results Obtained from the Male Subject

The analysis of the concentration curve of ^{35}[S]DS in plasma during the first 2 hours following the injection of the tracers (Fig. 4) by the standard procedure outlined under case 1 in the accompanying paper and other references therein results in two straight lines. Then, the disappearance of ^{35}S from DS in plasma may be described by two exponential terms. If $y_{DS}{}^{35}S$ denotes the ^{35}S content in the DS pool, then

$$y_{DS}{}^{35}S = C_1e^{-\alpha t} + C_2e^{-\beta t}$$

This equation corresponds to a two-pool system to which the equations shown under case 1 or 1a in the accompanying paper are applicable. The percentage of the dose of ^{35}S injected present in DS in 100 ml of plasma will therefore be expressed by the following equation:

$$\frac{10y_{DS}{}^{35}S}{R_{DS}{}^{35}SV_{DS}} = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t}$$

where V_{DS} is given in liters. Comparing the two equations above, $\lambda_1 = \alpha$, $\lambda_2 = \beta$, $A = 10C_1/R_{DS}{}^{35}SV_{DS}$, and $B = 10C_2/R_{DS}{}^{35}SV_{DS}$. The lines shown in Figure 4 were fitted to the data by the least squares method and correspond to the semilogarithmic plot of the two exponential terms. The line comprising the "tail" of the curve (samples F to I) gives the values $\lambda_2 = 0.166$ hr^{-1} ($t_{1/2} = 4.2$ hours) and $B = 1.99$. From the second straight line, $\lambda_1 = 5.0$ hr^{-1} ($t_{1/2} = 8.3$ minutes) and $A = 4.02$.

This result requires that two pools between which ^{35}S is transferred in both directions be considered. One, which includes the DS in plasma, is called the DS pool; the other is called the XS pool and may represent another space of distribution of DS or another steroid sulfate, i.e., the 3-monosulfate of androst-5-ene-3β,17β-diol. That situation is illustrated by the model in Figure 5.

The volume of the DS pool can be calculated according to the equation described under case 1 in the accompanying paper:

$$V_{DS} = \frac{R_{DS}{}^{35}S}{C_1/V_{DS} + C_2/V_{DS}} = \frac{10}{A + B} = 1.7 \text{ liters}$$

Since the plasma volume in this subject can be estimated from his weight to be about 4 liters, the low value of V_{DS} cannot be interpreted using the assumptions made. If DS bound to plasma proteins and unbound DS should be considered kinetically as two pools, then the procedure used to isolate DS from the blood samples gives a compound which represents both pools and the isotopic data obtained from DS isolated in this manner cannot be treated adequately. The values of the concentration of ^{35}S determined experimentally in plasma would represent an overestimation of the concentration of the ^{35}S in unbound DS and therefore the volume of the unbound DS pool would be underestimated. Further comments on this situation are presented under Discussion. The volume of distribution calculated from the extrapolation to time zero of the "tail" of the [^{35}S]DS concentration curve (Fig. 4) (as would be done if the existence of the XS pool and the distinction between bound and unbound DS in plasma are neglected) equals

$$\bar{V}_{DS} = \frac{10}{B} = 5.0 \text{ liters}$$

When two ^{35}S pools, the DS and the XS pool, are considered, rate constants are calculated in the following manner. Using the symbols shown in Figure 5 and the equations presented under case 1 in the preceding paper,

$$k_{DSXS} + k_{DSD} + k_{DS} = \frac{\alpha C_1 + \beta C_2}{R_{DS}{}^{35}S} = \frac{V_{DS}}{10}(A\lambda_1 + B\lambda_2) = 3.45 \text{ hr}^{-1}$$

and

$$k_{XSDS} + k_{XS} = \lambda_1 + \lambda_2 - (k_{DSXS} + k_{DSD} + k_{DS}) = 1.72 \text{ hr}^{-1}$$

The values of the rate constants of transfer between the DS and XS pools cannot be estimated from the available experimental data. However these constants can be calculated if it is assumed that all the material leaving the XS pool goes to the DS pool (Tait *et al.*, 1961; case 1a in the accompanying paper). This assumption would be valid if the XS pool represents a space of distribution of DS in which DS is not metabolized. However this assumption would not be tenable if XS represents a pool consisting of androst-5-

ene-3β,17β-diol sulfate, since it is known (J. Cos, E. Gurpide, and S. Lieberman, paper in preparation) that this diol is further metabolized and excreted. If the XS pool represents merely another space of distribution of DS, then it may be reasonable to assume that $k_{XS} = 0$. Then

$$k_{XSDS} = 1.72 \text{ hr}^{-1}$$

Also

$$k_{DSD} + k_{DS} = \frac{\lambda_1\lambda_2}{k_{XSDS}} = 0.48 \text{ hr}^{-1}$$

and

$$k_{DSXS} = (k_{DSXS} + k_{DSD} + k_{DS}) - (k_{DSD} + k_{DS}) = 2.97 \text{ hr}^{-1}$$

It should be noted that the value of $k_{DSD} + k_{DS}$ (0.48 hr^{-1}) is considerably larger than that obtained from the slope of the "tail" of the curve in Figure 5. This latter value would have been mistakenly taken to represent $k_{DSD} + k_{DS}$ had the existence of the XS pool (made evident only by the early part of the curve) been neglected.

The data in Table III show that during the time when blood samples were collected (2 hours) [^{3}H]DS and [^{35}S]DS appeared to have followed the same fate. The faster removal of [^{35}S]DS by reversible conversion of DS to D becomes apparent in experiments of longer duration such as that performed on the female subject. Furthermore, the small interval during which the blood samples were taken from the male subject was obviously insufficient to allow the precise determination of the slope of the "tail" of the curve in Figure 4.

The volume of the XS pool could be estimated if it is further assumed that al the material entering the XS pool comes from the DS pool, in which case

$$V_{XS} = \frac{k_{DSXS}}{k_{XSDS}} V_{DS} = 2.94 \text{ liters}$$

Discussion

One striking result of this study is the small volume of distribution of DS, the value estimated being considerably smaller than those of other steroids estimated by similar techniques. For instance, cortisol and aldosterone have been reported as having volumes of distribution of 15 liters (Peterson and Wyngaarden 1956) and 40 liters (Tait *et al.*, 1961), respectively. The estimated volume of distribution of DS indicates that the rapid mixing of the injected tracer (as well as the secreted DS) may be confined to only the vascular space. In the experiment on the male subject, a second space in which DS introduced into circulation distributes more slowly appeared to exist. The influence of the existence of a second sulfate pool exchanging with DS in plasma on the values calculated from the data obtained from the female subject was evaluated by analyzing mathematically the three-pool model in Figure 5 with respect to the injected tracers (J. Mann and E. Gurpide, unpublished results). From that analysis it was concluded that the slope of the [^{35}S]DS curve in Figure 3 represents a value *smaller* than the sum of all rate constants of removal of DS from the pool into which the tracer was injected. Furthermore, the value of V_{DS} calculated by the extrapolation of such a line to time zero, as described for the female subject, would result in an *overestimation* of that parameter. However, the low metabolic clearance rate of DS (approximately 7 liters/day as compared with 1600 liters/day for aldosterone (Tait *et al.*, 1962) and 3000 liters/day for progesterone (Little *et al.*, 1963) would suggest that endogenously produced DS mixes completely with all the existing DS before appreciable metabolism occurs. This follows since a low metabolic clearance rate indicates that a volume of blood will circulate many times before it is cleared of DS. Thus the assumption implicit in the model in Figure 1 of a single space of distribution of DS may be a tenable simplification.

Another assumption is that labeled sulfate, once cleaved from DS, is not reused to a significant extent in any subsequent sulfation of D. This is probably true considering the large dilution the radioactive sulfate ion would suffer in the body fluids. Furthermore, this assumption is supported by the findings of Baulieu *et al.* (1963) who found that after administration of [^{35}S]DS, urinary androsterone and etiocholanoline sulfates were unlabeled. Still another assumption upon which the model depicted in Figure 1 depends is that "indirect" pathways of metabolism of D and DS do not exist. The most likely example of such an indirect path is one involving androstenediol (ADL) and its sulfate (ADLS), as for example DS $\rightleftarrows$ ADLS $\rightleftarrows$ ADL $\rightleftarrows$ D. If such a path did exist, as has already been indicated (Baulieu *et al.*, 1963), it would influence the reality of the parameters calculated in this study. Recently in this laboratory J. Cos, E. Gurpide, and S. Lieberman (paper in preparation) have studied in a quantitative manner the conversion of D to DS and the conversion of DS to D by the pathway involving ADL and its sulfate. They found that only 5% of the transfer of D to DS and 20% of the transfer of DS to D occurs by the indirect pathway. Thus, the transfers via ADL and its sulfate appear to be of minor importance. From this point of view, therefore, the model in Figure 1 appears to be adequate. The experimental results obtained in this study do not afford evidence in support of a single space of distribution of the free compound, D. The short time necessary for the ^{14}C injected as the D tracer to reach its maximum concentration in DS in plasma (23 minutes in the male subject) indicates a large rate constant of metabolism of D. The metabolic clearance rate of D has been estimated as 2400 liters/day (Horton and Forsham, 1963). Just as a low metabolic clearance rate is consistent with a single space of distribution, so a high metabolic clearance rate, as appears to exist for D, makes it unlikely that secreted D (or the injected tracer) would distribute itself in the whole vascular system before being metabolized to a significant extent. On the other hand, following the reasoning presented by Tait *et al.* (1962) for aldosterone, a large metabolic clearance rate, i.e., one that approximates the blood flow through the liver, could mean that D is completely metabolized in one passage through that organ. If this were the case, then the D pool represented in Figure 1 would correspond to D in the liver and the rate r_{DDS} would be a measure of the sulfation process occurring therein.

As pointed out in the accompanying paper, the values of the production rate of DS in *blood* and the metabolic clearance rate of plasma DS as determined in the experiments on the female subject do not depend on the characteristics of the distribution and the metabolism of the compound. That is to say, the values estimated are independent of the number of pools exchanging with DS in plasma.

On the other hand, the estimated values of the rate constants and the rates of metabolism are dependent upon the model chosen. Also dependent on the model is the value of the volumes of distribution of DS as calculated in this study.

The reality of the values of the parameters calculated from *urinary* data is also dependent upon various as-

sumptions. For the calculation of the rates of secretion of D and of DS, it has been necessary to assume only (E. Gurpide and J. Mann, paper submitted for publication) (a) that urinary DG and DS have as their sole precursors D and DS secreted into circulation, and (b) that the metabolic fate of the tracers and the corresponding endogenously secreted compounds are identical. However, the calculation of the rates of transfer of D and DS is based on a model in which it is implied that the two compounds exchange directly with each other and that this exchange occurs only in the space receiving all the injected tracers. Furthermore, the urinary metabolites are assumed to be uniquely derived from each of the compounds present in that space. If the compounds do not mix rapidly throughout the whole body and the urinary metabolites are formed in several tissues, various rates, including the production rates, calculated from the specific activities of the urinary metabolites, will lack meaning. This is also true even if the metabolites are formed in only one tissue but their corresponding precursors are metabolized to other products in other tissues. The value of the production rates should always refer to a definite space and in the example given above, the values calculated from urinary data will refer to no specific spaces.

The results obtained from the urine samples of the female subject confirm the previous findings (VandeWiele *et al.*, 1963) that DS is secreted. In this subject the ratio of the secretory rates of DS and D is 2.6 and it is noteworthy that this is similar to the ratio of the differences in the concentrations of DS and of D found by Wieland *et al.* (1963) in adrenal venous blood and peripheral blood.

As carried out in this study, the calculation of the rates of secretion and of metabolism requires the use of formulas involving several experimentally determined values. Each of these values has associated with it some degree of imprecision, and when these values are used to calculate various metabolic parameters using the mathematical expressions given, the accumulation of many errors introduces uncertainty into the results. For instance, it has been estimated that the variance of the rate of secretion of DS, calculated using the average value of the various counting and colorimetric procedures, is approximately 30%. From this it is evident that it is not possible to determine whether the values obtained by the analysis of blood constituents are significantly different from those obtained by sampling urinary metabolites. The similarity of the values of PR_{DS} obtained from plasma and those obtained from urine does not constitute evidence supporting the model since the specific activity of urinary DS yields the same information as that obtained from the specific activity of DS in plasma (Tait, 1963; Gurpide *et al.*, 1964).

From the concentration of DS in plasma and the rate of urinary excretion of DS in the female subject the renal clearance rate of DS(C_{DS}) can be calculated since, by definition,

$$C_{DS} = \frac{u \times v}{p}$$

where u is the concentration of DS in urine (μmole/ml), p is the concentration of DS in plasma (μmole/ml), and v is the rate of formation of urine (ml/min).

Then,

$$C_{DS} = \frac{100}{1440} \cdot \frac{\epsilon_{DS(\mu mole/day)}}{c_{DS(\mu mole/100\ ml)}} = 0.03 \text{ ml/minute}$$

Bongiovanni and Eberlein (1957) and Kellie and Smith (1957) have both previously reported that the renal clearance of DS is small. The low clearance rate has been interpreted to indicate extensive binding of DS to plasma proteins. The binding of DS to albumin has been demonstrated and measured by Puche and Nes (1962).

As previously mentioned under Calculations, part B, the existence of a reversible process of binding of DS to plasma proteins may result in two kinetic pools of DS in blood. This situation would prevail if both bound and unbound DS are circulating and if the mixing of the two forms (as reflected by identical specific activities of DS in both forms) does not occur at a rate greater than the rate of metabolism of DS in either form. Such a situation, which could also exist for other steroids, would be difficult to cope with experimentally because the separation and measurement of both free and bound forms would be necessary.

Added in Proof

To the same female subject a mixture of ammonium [^{35}S]sulfate and [^{3}H]DS in a ^{3}H/^{35}S ratio of 0.1was administered. The DS isolated from a 3 day collection of urine showed an isotope ratio of 112.

References

Baulieu, E. E. (1962), *J. Clin. Endocrinol. Metab. 22*, 501.

Baulieu, E. E., Carpechot, C., and Emiliozzi, R. (1963), *Steroids 2*, 429.

Baulieu, E. E., and Dray, F. (1963), *J. Clin. Endocrinol. Metab. 23*, 1298.

Bongiovanni, A. M., and Eberlein, W. R. (1957), *J. Clin. Endocrinol. Metab. 17*, 238.

Burstein, S., and Lieberman, S. (1958), *J. Biol. Chem. 233*, 331.

Bush, I. E. (1952), *Biochem. J. 50*, 370.

Calvin, H., and Lieberman, S. (1964), *Biochemistry 3*, 259.

Calvin, H., VandeWiele, R. L., and Lieberman, S. (1963), *Biochemistry 2*, 648.

Cohn, G. L., Mulrow, P. J., and Dunne, V. C. (1963), *J. Clin. Endocrinol. Metab. 23*, 671.

Crepy, O. (1960), *Rev. Franc. Etudes Clin. Biol. 5*, 284.

Edwards, R. W., Kellie. A. E., and Wade, A. P. (1953), *Mem. Soc. Endocrinol. 2*, 53.

Gamble, J. L. (1952), Extracellular Fluid, Cambridge, Mass., Harvard University Press.

Gurpide, E., Angers, M., VandeWiele, R. L., and Lieberman, S. (1962), *J. Clin. Endocrinol. Metab. 22*, 935.

Gurpide, E., MacDonald, P. C., VandeWiele, R. L., and Lieberman, S. (1963), *J. Clin. Endocrinol. Metab. 23*, 346.

Gurpide, E., Mann, J., and Sandberg, E. (1964), *Biochemistry 3*, 1250 (this issue; accompanying paper).

Horton, R., and Forsham, P. H. (1963), Abstracts of the 45th Meeting of the Endocrine Society.

Kellie, A. E., and Smith, E. R. (1957), *Biochem. J. 66*, 490.

Lakshmanan, T. K., and Lieberman, S. (1954), *Arch. Biochem. Biophys. 53*, 258.

Little, B., Bougas, J., Tait, J. F., and Tait, S. A. S. (1963), Abstracts of the 45th Meeting of the Endocrine Society.

MacDonald, P. C., Gonzalez, O., VandeWiele, R. L., and Lieberman, S. (1963), *J. Clin. Endocrinol. Metab. 23*, 665.

McKenna, J., and Norymberski, J. K. (1960), *Biochem. J. 76*, 60P.

Migeon, J. C. (1963), *Federation Proc. 22*, 468.

Okita, G. J., Karaba, J. J., Richardson, F., and LeRoy, G. V. (1957), *Nucleonics 15*, 111.

Peterson, R. E., and Wyngaarden, J. B. (1956), *J. Clin. Invest. 35*, 552.

Puche, R. C., and Nes, W. R. (1962), *Endocrinology 70*, 857.

Roberts, K. D., VandeWiele, R. L., and Lieberman, S. (1961), *J. Biol. Chem. 236*, 2213.

Siiteri, P. K., VandeWiele, R. L., and Lieberman, S. (1963), *J. Clin. Endocrinol. Metab. 23*, 588.

Siiteri, P. K., and MacDonald, P. C. (1963), *Steroids 2*, 713.

Tait, J. F. (1963), *J. Clin. Endocrinol. Metab. 23*, 1285.

Tait, J. F., Little, B., Tait, S. A. S., and Flood, C. (1962), *J. Clin. Invest. 41*, 2093.
Tait, J. F., Tait, S. A. S., Little, B., and Laumas, K. R. (1961), *J. Clin. Invest. 40*, 72.
VandeWiele, R. L., MacDonald, P. C., Gurpide, E., and Lieberman, S. (1963), *Recent Progr. Hormone Res. 19*, 275.
Wallace, E. Z., and Lieberman, S. (1963), *J. Clin. Endocrinol. Metab. 23*, 90.
Warren, J. C., and Timberlake, C. E. (1962), *J. Clin. Endocrinol. Metab. 22*, 1148.
Wieland, R. G., Levy, R. P., Katz, D., and Hirschmann, H. (1963), *Biochim. Biophys. Acta 78*, 566.
Zimmermann, W. (1936), *Z. Physiol. Chem. 245*, 47.

Mechanisms of Steroid Oxidation by Microorganisms*

VI. Metabolism of 3β-Hydroxy-5α,6α-oxidoandrostan-17-one

S. S. Lee and Charles J. Sih

From the School of Pharmacy, University of Wisconsin, Madison

Received April 10, 1964

When 3β-hydroxy-5α,6α-oxidoandrostan-17-one (IV) was exposed to *Nocardia restrictus*, 6α-hydroxyandrost-4-ene-3,17-dione (VI) and 3,6*S*-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (V), mp 145–147°, $[\alpha]_D^{25}$ +13°, λ_{max}^{alc} 280 mμ (ε, 2400), λ 3.01, 5.75, 5.84, 6.21, 6.32, and 6.70 μ (KBr) were obtained. 3β,5α,6β-Trihydroxyandrostan-17-one (IX) was transformed into 6β-hydroxyandrost-4-ene-3,17-dione (X) and 3,6*R*-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (XI), mp 98–100°, $[\alpha]_D^{25}$ +3°, λ_{max}^{alc} 280 mμ (ε, 2300), λ 2.98, 5.78, 5.84, 6.20, 6.31, and 6.70 μ (KBr) by the same organism. The absence of a 6α-hydroxylase in *N. restrictus* was established by isotopic experiments. The mechanism of epoxide opening appears to involve the oxidation of compound IV into 5α,6α-oxidoandrostane-3,17-dione (XII), which can undergo nonenzymatic rearrangement to yield compound VI. A convenient chemical procedure for the preparation of compound VI is described.

Our continued interest in defining the degradative pathway of androst-4-ene-3,17-dione (I) by *Nocardia restrictus* prompted us to search for more suitable methods for the preparation of substantial quantities of 9α-hydroxyandrost-4-ene-3,17-dione (II), a starting material for the synthesis of other degradative intermediates. Previous studies have shown that the initial degradative reactions of compound I by *N. restrictus* involve 9α-hydroxylation followed by 1,2-dehydrogenation or vice versa to yield 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (III) (Dodson and Muir, 1961). However, A-norandrost-4-ene-3,17-dione was converted into 9α-hydroxy-A-norandrost-4-ene-3,17-dione in good yields and the latter product was not further metabolized at a significant rate (Sih, 1962). On the basis of this result, it appeared that if one could prevent or delay the formation of a $\Delta^{1,4}$-dienone system in ring A, it might be possible to obtain 9α-hydroxysteroids in good yields. The readily available compound, 3β-hydroxy-5α,6α-oxidoandrostan-17-one (IV) was selected to test this hypothesis. Although no substantial quantities of the desired 9α-hydroxysteroid accumulated, we would like to report our observations on the metabolism of compound IV by *N. restrictus* and the mechanism of the opening of the epoxide in compound IV.

When compound IV was exposed to *N. restrictus* two products were formed. The first product, compound VI, was characterized as 6α-hydroxyandrost-4-ene-3,17-dione on the basis that its infrared spectrum was identical to an authentic sample and a mixed-melting-point determination gave no depression. The second product, compound V, was initially recognized as a phenol by virture of its ultraviolet spectrum λ_{max}^{alc} 280 mμ (ε, 2400). Its infrared spectrum showed bands at 3.01 μ (OH), 5.75 μ (5-membered ring ketone), 5.84 μ (6-membered ring ketone), 6.21, 6.32, and 6.70 μ (aromatic ring). Carbon-hydrogen analysis afforded figures consistent with $C_{19}H_{24}O_4$. Methylation of compound V with methyl iodide and potassium carbonate in acetone afforded its corresponding methyl ether, compound VII, whose NMR[1] spectrum showed bands at 9.02 τ (3 H, 18-Me); 7.75 τ (3 H, 19-Me); 6.23 τ (3 H, CH_3-O); 4.65 τ (1H, J's 8, 10 cps 6-H); 2.92, 3.05, 3.27 and 3.42 τ (two vicinal protons on aromatic ring); 2.75 τ (proton at C-4 on aromatic ring). Oxidation of compound VII with pyridine–chromic acid afforded 3-methoxy-9,10-secoandrosta-1,3,5(10)-triene-6,9,17-trione (VIII) on the basis of the following data: Carbon-hydrogen analysis was in good agreement with $C_{20}H_{24}O_4$; its ultraviolet spectrum showed bands at 237 mμ (ε, 7200) and 308 mμ (ε, 3100), characteristic of methoxyindanones; its infrared spectrum showed bands at 5.76, 5.85, 5.90, 6.22, 6.31, and 6.71 μ. As expected, compound VI was converted into V by exposure to *N. restrictus* via 9α-hydroxylation and 1,2-dehydrogenation. All these results suggest that the epoxide in compound IV had been opened and the structure of compound V is 3,6*S*-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (Cahn *et al.*, 1956).

Several examples of microbiological opening of steroid epoxides have been observed. Camerino *et al.* (1956) observed the conversion of 16α,17α-oxidopregn-4-ene-3,20-dione into 16α,20α-dihydroxy-17β-methyl-18-nor-17α-pregna-4,13-dien-3-one by yeast via a retropinacoline-type rearrangement. Camerino and Sciaky (1959) reported the transformation of 4β,5β-oxidopregnane-3,20-dione into 3β,4β,5α-trihydroxypregnan-20-one by yeast. Prochazka *et al.* (1961) noted that 3β-hydroxy-5,6-oxido-B-norandrostan-17-one was transformed into 3β,5α,6β-trihydroxy-B-nor-

* This investigation was supported in part by U. S. Public Health Service research grants (AM 04874 and AM 06110). For paper V of this series see Tsong *et al.* (1964).

[1] Abbreviation used in this work: NMR, nuclear magnetic resonance.