Studies on the Metabolism of Blood-Borne Cholesterol Sulfate*

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ABSTRACT: Using isotopic dilution techniques, certain metabolic properties of cholesteryl 3β-sulfate [cholesterol sulfate (CS)], a substance that has recently been isolated from human blood and adrenal tissue, were studied in two normal human subjects. Thus, the concentration (c) of circulating CS^- in the plasma was found to be 328 and 924 μg/100 ml. Following the injection of [^{14}C]CS, the metabolic clearance rate (MCR) of this compound was determined from the disappearance curve of the tracer from plasma. From these data, the half-life was calculated to be 14–15 hr in these subjects. The production rate of cholesterol sulfate in plasma (PR = MCR·c) was found to be 35 mg/day in one subject and 163 mg/day in the other. In other experiments, in which mixtures of [^{14}C]CS and either [^{3}H]cortisol or [^{3}H]dehydroisoandrosterone sulfate were injected, little or no conversion of the sterol sulfate to these two major products of the human adrenal gland was detected. This conclusion was reached by the examination of the ^{3}H/^{14}C ratios in the appropriate urinary metabolites. The major metabolic fate of the administered cholesterol sulfate tracer was its conversion to free and to fatty acid esterified cholesterol.

Cholesterol sulfate (CS)[1] has recently been shown by Drayer *et al.* (1964) to be a naturally occurring entity. This conjugate has been found in bovine adrenal tissue, human blood (Drayer *et al.*, 1964), and adrenal tumor tissue (N. M. Drayer, unpublished data). Recently, Moser *et al.* (1966) also isolated this sulfate from natural sources. That CS may serve, too, as a substrate for the mitochondrial side-chain cleaving enzyme of bovine adrenal tissue has been described by Raggatt *et al.* (1965). Moreover, Roberts *et al.* (1964) perfused CS through a human adrenal adenoma, *in situ*, and the results suggest that CS was converted to a variety of Δ^5-3β-sulfated steroids *via* intermediates which retain the sulfate grouping. The purpose of the present study was to determine the plasma concentration of CS, the rate at which blood-borne CS is produced (PR_{CS}), and the quantitative importance of circulating CS as a precursor of steroids. Values for the metabolic clearance rate, MCR, were obtained by injecting ^{14}C-labeled CS intravenously and measuring the concentration of radioactivity present as CS in plasma samples withdrawn at various intervals following the injection of the tracer. Rates of production of CS in plasma were obtained by multiplying the MCR by the plasma concentration (PR_{CS} = MCR·c). In order to determine whether CS is converted to cortisol, a mixture of [^{14}C]CS and [^{3}H]cortisol was injected into a patient with an adrenal tumor. The specific activity with respect to ^{14}C of urinary tetrahydrocortisol indicated that little or none of the cortisol secreted by the adrenals was derived from blood-borne CS. In a normal subject, an analagous experiment using [^{14}C]CS and [^{3}H]DS led, also, to the conclusion that none of the DS produced in the adrenal arose from circulating CS.

Experimental Section

Measurement of Radioactivity. ^{14}C and ^{3}H were measured simultaneously in a liquid scintillation counter (Packard Instrument Model 3003). Depending upon the solubility characteristics of the samples, they were counted either in a toluene solution of PPO and POPOP or in a mixture of 2 ml of methanol and 10 ml of the toluene solution. The gains for the three channels of the instrument were set to provide maximum efficiency for tritium in channel 1, for ^{14}C in toluene solutions in channel 2, and ^{14}C in toluene–MeOH solutions in channel 3. The optimal gain for ^{3}H was only slightly affected by the addition of methanol to the toluene solution. The efficiencies for counting ^{3}H and ^{14}C in toluene solutions were, respectively, 31 and 11% in channel 1 and 0.5 and 60% in channel 2, and for counting in toluene–MeOH solutions, 20 and 23% in channel 1, and 0.1 and 49% in channel 2. Corrections for changes in efficiency were made. Corrections for quenching were performed by the addition of internal standards, as previously described (Sandberg *et al.*, 1964). In this paper, all data on radioactivity is

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[1] The following trivial names and abbreviations have been used in the text: cholesterol sulfate (CS), cholesteryl 3β-sulfate; cortisol (compound F), 11β,17α,21-trihydroxy-4-pregnen-3,20-dione; dehydroisoandrosterone (D), 3β-hydroxy-5-androsten-17-one; dehydroisoandrosterone sulfate (DS), 3β-hydroxy-5-androsten-17-one 3-sulfate; tetrahydrocortisol (THF), 3α,11β,17α,-21-tetrahydroxypregnan-20-one; tetrahydrocortisone (THE), 3α,17α,21-trihydroxypregnan-11,20-dione; androsterone sulfate (AS), 3α-hydroxyandrostan-17-one 3-sulfate; etiocholanolone sulfate (ES), 3α-hydroxyetiocholan-17-one 3-sulfate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

TABLE I: Chromatographic Systems.

	System	Composition
Celite columns	C-1	Isooctane–ethyl acetate–*t*-butyl alcohol–methanol–1 M ammonia, 5:1:2:2:3
	C-2	Isooctane–ethyl acetate–*t*-butyl alcohol–methanol–1 M ammonia, 2:4:2:2:3
	C-3	Isooctane–toluene–methanol–water, 2:2:4:1 (reversed phase)
	C-4	*n*-Heptane–1-butanol–methanol–1 M ammonia, 4:0.7:1.5:2
	C-5	*n*-Heptane–1-butanol–methanol–0.6M pyridinium chloride–pyridine, 4:1:2:2:0.1
	C-6	Benzene–methanol–water, 4:2:1 (Celite:stationary phase, 4:1 g/ml)
	C-7	Isooctane–ethyl acetate–methanol–water, 30:10:14:6
	C-8	Isooctane–ethyl acetate–methanol–water, 40:20:40:10
	C-9	Isooctane–ethyl acetate–methanol–water, 100:50:75:75
Thin layer plates (silica gel "G")	TLC-1	Butanone–ethanol–benzene–water, 3:3:3:1
	TLC-2	Benzene–ethyl acetate, 2:1
	TLC-3	*n*-Heptane–ethyl acetate, 9:1
Paper	P-1	Water–propanol–kerosene, 4:6:1 (reversed phase)
	P-2	Benzene–methanol–water, 2:1:1
	P-3	Isopropyl ether–hexane–*t*-butyl alchol–1 M ammonia, 5:2:3:10
	P-4	Hexane–methanol–water, 100:85:15
	P-5	Ethylene chloride–formamide
	P-6	Benzene–methanol–water, 4:2:1
	P-7	Heptane–methanol–water, 100:95:5

expressed as disintegrations per minute (dpm) for convenience in comparing measurements obtained at different efficiencies.

Chromatography. Partition chromatography on Celite was performed as described by Siiteri (1963), unless otherwise indicated. Plates for thin layer chromatography were prepared from a slurry of one part silica gel "G" (Merck and Co.) and two parts water. Woelm alumina (6% water) was used for column chromatography. Whatman No. 1 paper was used as a support for paper chromatography. Solvent systems for the various chromatographic procedures are presented in Table I.

Preparation and Purification of Tracers. SODIUM [7α-^{3}H]CHOLESTEROL SULFATE. The preparation and purification of this tracer has been described previously (Roberts *et al.*, 1964).

AMMONIUM [4-^{14}C]CHOLESTEROL SULFATE. [4-^{14}C]-Cholesterol was obtained from New England Nuclear Corp. and purified by paper chromatography (system P-1; Martin, 1957) without addition of carrier. Its specific activity, as reported by the manufacturer and verified in this laboratory, was approximately 400,000 dpm/μg. The labeled cholesterol was sulfated and the sulfate was purified as described for the preparation of ammonium [7α-^{3}H]cholesterol sulfate (Roberts *et al.*, 1964). A mixture of the ^{3}H- and ^{14}C-labeled CS was chromatographed on Celite (system C-1) and the ratio of the isotopes was found to be constant throughout the single peak of radioactivity that was obtained.

[1,2-^{3}H]CORTISOL. This tracer was purchased from Tracer Labs. The specific activity reported by the manufacturer was 50 μc/μg. It was purified by chromatography on paper (system P-2). Radiochemical purity was proved by crystallization from ethanol after the addition of carrier to an aliquot of the tracer solution (specific activities: crystals, 3070 dpm/mg; residue from mother liquor, 2970 dpm/mg).

AMMONIUM [7α-^{3}H]DEHYDROISOANDROSTERONE SULFATE. [7α-^{3}H] Dehydroisoandrosterone (1 mc), purchased from the New England Nuclear Corp., was chromatographed on paper (system P-4) for 7 hr. The radioactive band was located by radioautography and was eluted with methanol. Sulfation was performed according to the procedure already described (Roberts *et al.*, 1964). Purification of the labeled conjugate was achieved by chromatography on paper (system P-3; Baulieu, 1963) for 24 hr. Further purification was achieved by chromatography on Celite (system C-2). An aliquot of this purified material was solvolyzed and chromatographed on paper (system P-4) for 7 hr. One radioactive band corresponding in mobility to authentic dehydroisoandrosterone was obtained. The radiochemical homogeneity was verified by crystallizing a portion of the tracer with carrier ammonium DS.

Subjects. Subjects 1 and 2 were normal males, 35 and 34 years old, respectively. Subject 3 was a 17-year-old girl with an adrenal tumor and liver metastases. At the time of the study, the patient exhibited the features of Cushing's syndrome but received no treatment. A clinical report on this patient has been presented by Roginsky and Schick (1966).

Administration of Tracers and Collection of Samples. Subject 1 was injected intravenously with 4.5×10^6 dpm of [4-^{14}C]CS (13 μg). Prior to the injection of the tracer, a blood sample was withdrawn to determine the plasma concentration of CS. After administration

TABLE II: Postinjection Samples from Subjects 1 and 2.

Sample	Subject	Time after Injection [^{14}C]CS (hr)	Vol. of Plasma Sample (ml)	[^{3}H]CS Added to Plasma Sample (dpm)	^{3}H/^{14}C in CS (dpm/dpm)	% of Dose/l. of Plasma
I	1	2	21	54,800	10400/2050 = 5.1	11.5
	2	2	26	87,500	7800/70 = 10.2	6.9
II	1	8	23	36,500	1700/290 = 5.8	5.9
	2	8	27	64,500	2540/175 = 14.5	3.4
III	1	25	21	29,300	7700/880 = 8.8	3.5
	2	26	25	35,000	6700/425 = 15.8	1.8
IV	1	49	23	29,300	7800/285 = 27.3	1.0
	2	50	26	16,500	2720/140 = 19.4	0.7
V	1	73	47	58,500	18500/270 = 68.5	0.4
	2	74	50	16,500	320/16 = 20	0.3

of the tracer, blood samples were taken at various intervals up to 73 hr (Table II).

Subject 2 was injected intravenously with 4.8×10^6 dpm of [4-^{14}C]CS (14 μg) and 0.70×10^6 dpm of [7α-^{3}H]DS (0.1 μg). Blood samples were withdrawn prior to and at various intervals up to 74 hr following injection of the tracers (Table II). During the experiment, daily urine specimens were collected separately for 10 consecutive days after the injection.

Subject 3 was injected intravenously with a mixture of 16.7×10^6 dpm of [4-^{14}C]CS (51 μg) and 3.2×10^6 dpm of [1,2-^{3}H]cortisol (0.03 μg). Blood samples were withdrawn before the injection and at different intervals during 72 hr following the injection. Urine was collected for 9 days; each daily specimen was examined separately.

Analysis of Blood Specimens from Normal Male Subjects 1 and 2. POSTINJECTION SAMPLES. In order to estimate procedural losses, a tracer quantity of tritiated cholesterol sulfate dissolved in approximately 0.5 ml of ethanol was mixed with each sample of plasma obtained after centrifugation of the blood. About 0.5 hr later, the plasma was poured with stirring, into three times its volume of freshly distilled tetrahydrofuran. After having been stirred for 10 min, the precipitated proteins were filtered, washed with tetrahydrofuran, and discarded. Solid sodium chloride was added to the filtrate until two phases appeared. The lower aqueous phase was discarded. The upper layer was taken to dryness and chromatographed on Celite (system C-3) using a reversed-phase technique. The support Celite (30 g), was mixed with the upper phase of the solvent system (0.4 ml of solvent/g of Celite).

In order to elute the sulfates, approximately 30 ml of the lower phase was passed through the column. An aliquot of this eluate was counted in order to establish the amount of tritiated cholesterol sulfate to be added to the subsequent plasma samples to obtain a ^{3}H:^{14}C ratio acceptable for efficient counting. The preferred ratio ranges from 2:1 to 10:1. The residue obtained after evaporation of the remainder of the eluate to dryness, was dissolved in 0.5 M pyridinium sulfate solution and extracted twice with two volumes of chloroform (McKenna and Norymberski, 1960). Ammonia was added to the organic phase, prior to evaporation, in order to obtain ammonium salts in the dry residue. The residue was chromatographed twice (system TLC-1; Wusteman *et al.*, 1964) on 20-cm plates and the area corresponding to CS (R_F 0.5) was eluted with methanol. The eluted material was solvolyzed (Burstein and Lieberman, 1958) and the liberated cholesterol was purified by thin layer chromatography (system TLC-2). The zone corresponding to standard cholesterol (R_F 0.65) was eluted and chromatographed in the reversed-phase paper system (system P-1, R_F 0.58) of Martin (1957) for 20 hr. An aliquot of the eluate from paper was counted to determine the ^{3}H/^{14}C ratio. Further purification of the sterol was achieved by acetylation and chromatography on system P-1 (R_F of cholesterol acetate 0.17).

To obtain the free and esterified cholesterol remaining on the reversed phase Celite column (C-3) after the elution of the sulfate fraction, the column was washed with benzene–methanol (1:1) and benzene. The combined washes were taken to dryness and the residue was chromatographed on system TLC-2. This afforded a separation of cholesterol from its esters; the latter moved with the solvent front. The cholesterol fraction was rechromatographed on the same system. The cholesterol esters were saponified with 5% methanolic potassium hydroxide by refluxing for 2 hr. The free cholesterol, extracted with hexane, was purified by chromatography on alumina (eluted with hexane–benzene, 3:1). The weight of cholesterol, in the specimens used for the estimation of specific activities, was determined using the Liebermann–Burchard method.

PREINJECTION SAMPLES. To determine the concentration of CS in plasma, a tracer quantity of [7α-^{3}H]CS was added to a known volume of plasma (Table II).

TABLE III: Calculation of Plasma Concentrations of CS^- in Subjects 1 and 2.

Subject	Vol. of Plasma	[^{3}H]CS Added to Plasma (dpm)	Sp Act. of [^{14}C]Ac_2O (dpm/μmole)	$^3H/^{14}C$ in Chol-acetate (dpm/dpm)	c_{CS^-} (μg/100 ml)
1	43	1.5×10^6	576,000	17.4	328
2	43	1.8×10^6	910,000	4.5	924

$$c_{CS^-} = \frac{200 \times 466R}{vra}$$

where R is dpm of [^{3}H]CS added to the plasma, 466 is the molecular weight of CS^-, v is volume of plasma to which [^{3}H]CS was added, r is $^3H/^{14}C$ ratio (dpm/dpm) in the isolated cholesterol acetate, and a is the specific activity (dpm/μmole) of [^{14}C]acetic anyhdride.

TABLE IV: Crystallization Data for the Estimation of the Concentration of Plasma CS^- in Subject 2.

		$^3H/^{14}C$ (dpm/dpm)		
		1	2	3
Cholesterol acetate	Crystals	4.4	4.3	4.5
	Mother liquor	0.65	3.3	4.1
Epoxide of cholesterol acetate	Crystals	4.8	—	—
	Mother liquor	4.8	—	—

The isolation and purification procedures for CS and for the cholesterol obtained after solvolysis of this conjugate, were the same as described above. In these instances, however, the cholesterol samples were acetylated with [^{14}C]acetic anhydride of known specific activity in order to establish the specific activity of the sterol by the double-isotope technique and, therefore, the weight of CS present in the original sample of plasma (Table III). The doubly labeled acetate was chromatographed either on system P-1 or on system TLC-3. To the eluate, 10 mg of carrier cholesterol acetate was added and the mixture was repeatedly crystallized from acetone until constant $^3H/^{14}C$ ratio was achieved. As a further test for radiochemical homogeneity, the product from the final crystallization was dissolved in 1 ml of chloroform which contained 10 mg of *m*-chloroperbenzoic acid. The solution was left overnight at room temperature, after which it was partitioned between chloroform and water. The chloroform-soluble residue was purified by chromatography on 2 × 15 cm paper strips (system P-1) (R_F 0.3). Following elution, 3β-acetoxycholesteryl-5,6-epoxide was crystallized from methanol–acetone. The $^3H/^{14}C$ ratios did not change appreciably during the above procedures. The crystallization data from subject 2 is presented in Table IV.

Analysis of Blood Specimens of Subject 3. POSTINJECTION SAMPLES. Ammonium cholesterol sulfate was added to blood samples obtained from subject 3 in order to correct for losses incurred during isolation procedures. Since subject 3 was the first subject studied, the rate of disappearance of the injected tracer was unknown. Thus, the quantity of [^{3}H]CS required to provide an appropriate $^3H/^{14}C$ ratio could not be estimated. Unlabeled CS was therefore added to correct for losses. Due to difficulties in dissolving the carrier in propylene glycol, it could not be ascertained whether complete mixing of the carrier and labeled CS was achieved. After precipitation of the protein with tetrahydrofuran, CS was isolated by chromatography on alumina and eluted with methanol–water (3:1). It was rechromatographed on Celite (system C-4) as described by Drayer *et al.* (1965). Further purification of CS in the specimens used for the estimation of the specific activities of CS was determined by the methylene blue colorimetric method of Roy (1956) as modified by Crepy and Rulleau-Meslin (1960).

The nonesterified cholesterol fraction eluted from the above-mentioned alumina column with chloroform–benzene (1:1), was chromatographed on system TLC-2. The specific activities of cholesterol were determined by using weights estimated by the Liebermann–Burchard method (Cook and Rattray, 1958).

PREINJECTION SAMPLE. [4-^{14}C]CS (612,000 dpm, 2 μg) was added to the heparinized blood sample which was withdrawn prior to the injection of the tracer.

After 30 min, plasma was separated by centrifugation and the proteins were removed by precipitation with tetrahydrofuran. The residue was rechromatographed on alumina as described above after the solvent had been evaporated. The sulfate fraction was eluted with methanol–water (3:1) and was rechromatographed on Celite (system C-4). The CS was rechromatographed on system C-5. The specific activity of CS was measured using the methylene blue reagent. The value obtained was verified as follows. The purified CS was solvolyzed (Burstein and Lieberman, 1958) and the liberated cholesterol was purified by chromatography on alumina. The fraction eluted with benzene was acetylated with [^{3}H]acetic anhydride of known specific activity and the product was chromatographed on alumina and eluted with hexane–benzene (3:1). Carrier cholesterol acetate was added and the mixture was recrystallized from acetone until the $^3H/^{14}C$ ratio in the product and in the material remaining in the mother liquor was equal. In this way, the concentration of CS in the preinjection sample was established.

Analysis of Urine Specimens. One-third of each daily specimen from subject 2 was processed separately for the isolation of THF and THE. Another one-third was used for the isolation of D. The remaining samples (days 1–10) were combined and processed for the isolation of THF, THE, and D.

One-third of each daily urine collection from subject 3 was processed separately for the isolation of THE and THF. Another one-third of each sample was combined and the pooled samples were analyzed for these metabolites.

Isolation of Dehydroisoandrosterone. Sufficient sodium chloride was added to each urine specimen so that the resulting mixture was a 20% solution. The urine was extracted with a volume of tetrahydrofuran equal to the original urine volume. The organic extract was filtered through glass wool and then solvolyzed according to the method of Burstein and Lieberman (1958). Following the solvolytic procedure, the residue remaining after evaporation of the solvent was taken up in ether and the ether was washed twice with 0.1 N NaOH.

The ether-soluble residue from the urine of subject 2 was chromatographed on paper (system P-7) for 12 hr after having been equilibrated for 4 hr. The material corresponding in R_F to authentic D was eluted and quantitated using the Zimmermann reagent. The identity of this substance was subsequently verified by infrared spectroscopy.

Isolation of Urinary Tetrahydrocortisone and Tetrahydrocortisol. Each daily urine specimen was processed as follows. The urine was adjusted to pH 5 with 50% H_2SO_4 and 0.1 M acetate buffer was added (5 ml/100 ml of urine). The mixture was incubated for 3 days at 37° after β-glucuronidase (Ketodase) had been added (30,000 units/100 ml of urine). Following the incubation period, the urine was extracted with a volume of ethyl acetate equal to the total volume of urine. The organic extract was washed twice with 0.1 N NaOH and with water to neutrality. The residue remaining after evaporation of the solvent was subjected to a benzene–water partition. The aqueous phase was extracted three times with ethyl acetate.

The residue remaining from the urine of subject 2 after the ethyl acetate had been evaporated was chromatographed on paper (system P-5) for 42 hr. The individual zones representing THE and THF were rechromatographed on paper (system P-6) for 17 hr after having been equilibrated for 6 hr. The THE and THF that were isolated from the pooled urine sample of subject 2 were processed further by acetylation and rechromatography on system TLC-2. In each case, the specific activities were determined using the Porter–Silber reaction for the estimation of weight (Porter and Silber, 1950).

The residue from the urine of subject 3 which remained after evaporation of the second ethyl acetate extraction, was chromatographed on Celite using system C-6 (O. Gonzalez, and W. Sherman, unpublished data). In this partition system, THE appeared in the third holdback volume and THF appeared in the fifth holdback volume. The weight of the eluted material was estimated using the Porter–Silber reagent and the specific activities were determined. The tetrahydro metabolites were then acetylated and the resulting acetates were crystallized to constant specific activity. Identity was established by infrared spectroscopy.

Results

Normal Male Subjects 1 and 2. Table III shows the $^3H/^{14}C$ ratios in CS present in each plasma sample. As described in the experimental section, these ratios were measured in the cholesterol isolated after solvolysis of the purified CS. No changes were observed in the isotope ratios when measured before and after acetylation of the cholesterol samples. Table II also shows the concentration of [^{14}C]CS in each of the samples (expressed as per cent of the injected dose per liter of plasma).

The semilogarithmic plot of these concentrations *vs.* the time elapsed between the injection and the sampling, is shown in Figure 1. The straight line which best fits the points for each of the subjects, was calculated by the method of least squares. The 74-hr value for subject 2, was not used because the low ^{14}C content (Table II) made the counting unreliable. The slope of the line gives the "half-life" ($t_{1/2}$) and the rate constant of disappearance of the tracer from plasma ($k = 0.693/t_{1/2}$). The intercept (A) of the extrapolated line with the ordinate gives the volume ($V = 100/A$) in which the tracer is distributed, assuming a single kinetic space of distribution of the tracer at a concentration equal to that in plasma. The values calculated in this manner are shown in Table V. Using these estimates, the metabolic clearance rates of CS in plasma (MCR = kV) were calculated. These are shown in Table V along with the concentrations of CS^- [2] in

[2] CS^- refers to the anionic species (*i.e.*, $ROSO_3^-$).

TABLE V: Parameters of CS^- in Two Normal Males.

Parameter	Subject 1	Subject 2
Rate constant of disappearance of CS from plasma (hr^{-1})	0.050	0.045
Half-life (hr)	14	15.5
Volume of distribution of CS (l.)	9.1	16.5
MCR of CS from plasma (l./day)	10.8	17.7
Concentration of CS^- in plasma (μg/100 ml)	328	924
Production rate of CS^- in plasma (mg/day)	35	163

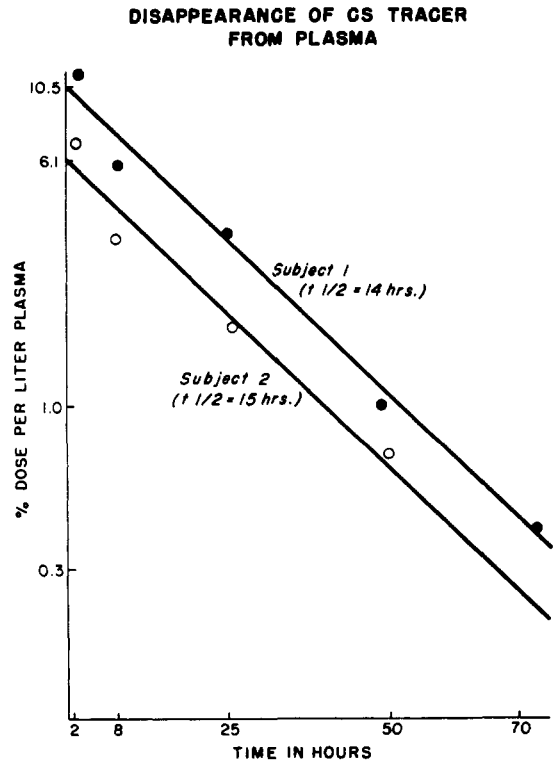


FIGURE 1.

plasma, calculated from the data presented in Table III, and the production rates of CS in plasma ($PR_{CS^-} = MCR \cdot c_{CS^-}$).

In the above calculations, a single space of distribution of CS and negligible rates of interconversion between CS and other compounds, were assumed. Since data on the disappearance of the tracer during the first 2 hr following the injection was not obtained, these assumptions were not actually tested. Examination of the graph in Figure 1 suggests that, for each subject, the 2-hr point may lie above the line determined by subsequent points. Although elimination of the first point in each curve results in half-lives of CS which do not exceed 18 hr, the influence of the existence of other pools of distribution of CS upon the validity of the calculations presented must be considered. As discussed in reference to a similar situation involving the metabolism of dehydroisoandrosterone sulfate (Sandberg *et al.*, 1964), the long half-life of the tracer observed after 2 hr indicates that the calculated values for MCR and PR would be affected only slightly by the existence of a second kinetic pool for CS^-. The MCR calculated for subject 1, assuming a single space of distribution for CS, is given by the reciprocal of the area under the curve ACD (Figure 2) (Tait, 1963), in which the experimentally determined portion of the curve (CD) is extrapolated to time zero (intercept A) and to the time when the plasma is practically devoid of tracer. On the other hand, the actual MCR may be given by the reciprocal of the area under the hypothetical curves BCD which would be obtained if experimental values for the 0–2-hr interval were included. Therefore, the relative error [(true MCR − calculated MCR)/true MCR] in the determination of MCR equals that fraction of the total area under curve BCD represented by area ABC. After 2 hr (or even earlier), the true curve is coincidental with ACD. Since the value of B is not greater than 50% of the injected dose per liter of plasma, it can be estimated, based on the analysis of the curve in Figure 2, that the maximum error inherent in the calculated MCR and therefore in PR is less than 20%. The maximum value for B assumes rapid mixing of the tracer in a vascular system containing 2 l. of plasma. On the other hand, if more than one kinetic space of distribution of CS actually exists, the values for the volume of rapid initial distribution calculated on the basis of a single kinetic space ($100/A$) may be much larger than the true value ($100/B$).

Both the free and the esterified cholesterol isolated from the plasma of subject 2 contained the isotope (^{14}C) associated with the injected CS tracer, thus indicating that circulating CS was converted *in vivo* to cholesterol. Some ^{14}C in the free cholesterol was derived by hydrolysis of CS during the isolation procedures rather than from *in vivo* processes. This follows from the fact that 1–2% of the tritium, which was added to the plasma as [^3H]CS, was found in the free cholesterol. The portion of ^{14}C present in free cholesterol that was derived as an artifact of hydrolysis, could be calculated from the ^3H content of the free cholesterol and from the $^3H/^{14}C$ ratio found in a sample of cholesterol sulfate isolated from the same plasma specimen. In the sample obtained 74 hr after the injection of the tracers, the specific activity with respect to ^{14}C, of the free cholesterol (corrected for the artifactitious hydrolysis of CS) was about 280 dpm/mg and that of cholesterol in the esterified fraction was about 50 dpm/mg.

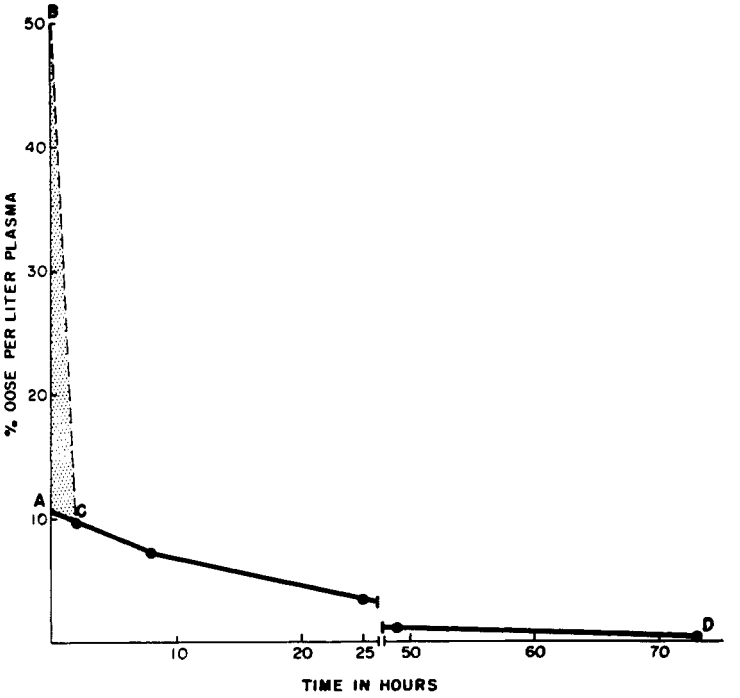


FIGURE 2: Curve of disappearance of labeled CS^- from plasma. C and D denotes portion of the curve obtained experimentally. A represents extrapolation of the experimental curve to $t = 0$, assuming a single space of distribution of CS^-. B shows percentage of dose per liter of plasma at $t = 0$, assuming that the injected tracer was rapidly distributed in 2 l. of plasma. From the graph, an estimate can be made of the maximal error introduced into MCR and PR_{CS}^- when only one pool of distribution is considered.

Subject 2 produced 8 mg of DS daily, as estimated by dividing the dose of [^{3}H]DS injected by three times the specific activity with respect to tritium, of urinary DS excreted during the first 3 days following the injection of the tracers. The DS, AS, and ES isolated from this subject's urine were devoid of ^{14}C (either in the samples from the daily specimens or in those from the pooled urine). Circulating CS did not serve as a significant precursor either of D or of DS in this normal individual. The specific activities of THF and of THE isolated from the first 10 days pooled urine sample of subject 2 were approximately 80 dpm/mg.

The fraction of a metabolite (or that of a hormone) originating from circulating CS can be estimated from available data through the following considerations. If the labeled tracer of CS were infused intravenously at a constant rate (P_{CS}) until constant values of the specific activity of CS in plasma (a_{CS}) and constant values of a metabolite of CS, in blood or in urine (a_{met}) were reached, then

$$\Delta_{CS,met} = \frac{a_{met}}{a_{CS}} \tag{1}$$

where $\Delta_{CS,met}$ is the fraction of the metabolite derived from circulating CS.

Since $a_{CS} = P_{CS}/PR_{CS}$ (Gurpide *et al.*, 1963), then

$$\bar{\Delta}_{CS,met} = \frac{a_{met}PR_{CS}}{P_{CS}} \tag{2}$$

In the same publication, it was shown that

$$\frac{a_{met}}{P_{CS}} = \frac{\bar{a}_{met}t}{R_{CS}}$$

where $\bar{a}_{met}$ is the specific activity of a metabolite isolated from urine which has been collected during a length of time, t, following the single intravenous injection of a dose, R, of labeled CS tracer. This expression is only valid when the value of t is large enough to allow for the complete excretion of the labeled urinary metabolite. It is evident that the above equation is true because each quotient, on either side of the equal sign, represents that fraction of the administered isotope which appears in the urinary metabolite. Equation 2 now can be written as

$$\bar{\Delta}_{CS,met} = \frac{\bar{a}_{met}tPR_{CS}}{R_{CS}}$$

The fraction of secreted F derived from blood-borne

CS was then calculated using the expression

$$\Delta_{CS,F} = \bar{a}_{THE} l \frac{PR_{CS}}{R_{CS}}$$

since these metabolites are considered to be derived only from cortisol. $\Delta_{CS,F}$, calculated in this manner, was 0.04. In subject 2, the fraction of circulating CS which is converted to cortisol ($\rho_{CS,F}$)[3] may be roughly estimated by assuming a secretory rate for F of 20 mg/day. If 4% of this rate were derived from circulating CS, then 0.8 mg/day or 0.5% of PR_{CS} was converted to F.

Subject 3. Analysis of the decline of the specific activity of cholesterol sulfate with time, after 2 hr following the injection of tracer into subject 3, indicates a half-life of CS^- of 18 hr. The concentration of CS^- in whole blood was 174 μg/100 ml and, assuming that no CS^- is carried by the blood cells, this is equivalent to 270 μg/100 ml of plasma. The production rate of CS^- in blood was estimated to be 4.4 mg/day. Unfortunately, as mentioned in the Experimental Section, there was some uncertainty concerning the adequacy of mixing of the carrier cholesterol sulfate with the labeled cholesterol sulfate contained in the blood samples. Incomplete mixing would have resulted in an underestimation of PR_{CS}. Obviously, this uncertainty does not apply to the estimates of the concentration of CS^- in the blood nor does it apply to the urinary data. In the plasma of subject 3, as well as in the plasma of subject 2, ^{14}C was found in both the free and the esterified fractions. The specific activity of free cholesterol in the last blood samples (74 hr) was 500 dpm/mg.

Labeled THE and THF were isolated from the pooled urine sample (9 days) and converted to their crystalline diacetates. The specific activities of these metabolites with respect to ^3H indicated a secretory rate of cortisol of 120 mg/day. The $^3H/^{14}C$ ratio was 37 (dpm/dpm), from which it could be estimated that 0.5% of the injected CS tracer was converted to circulating cortisol

$$\rho_{CS^-,F} = \frac{(^3H/^{14}C)_{injected}}{(^3H/^{14}C)_{THE}} \qquad (3)$$

The validity of this calculation depends upon the complete excretion of radioactivity. Actually, THE and THF isolated during the 9th day after injection, still contained ^{14}C introduced with the CS tracer. The daily values of the specific activities of THE and THF fluctuated between 80 and 160 dpm/mg. Therefore, $\rho_{CS^-,F}$ may have been underestimated. A 0.5% conversion of CS to cortisol and a production rate of CS^- in plasma of 4 mg, indicate that 20 μg/day of cortisol were formed from circulating CS. The comparison of this value with the estimated rate of secretion of cortisol (120 mg/day) leads to the conclusion that blood-borne CS was not a significant precursor of corticosteroids in this subject. As mentioned above, it cannot be concluded from the experimental data whether the calculated rate of production of CS was correct or whether it was grossly underestimated because of incomplete mixing with the added carrier. However, even if the production rate in this subject were similar to those found in subjects 1 and 2, the conclusion that circulating CS was not a significant precursor of cortisol would still be warranted.

[3] It may be valuable to call attention again to the difference between $\rho_{CS,F}$ and $\Delta_{CS,F}$. $\rho_{CS,F} = r_{CS,F}/PR_{CS}$, where $r_{CS,F}$ is the rate of conversion of circulating CS to circulating F and PR_{CS} is the rate of production of blood-borne CS. On the other hand, $\Delta_{CS,F} = r_{CS,F}/PR_F$, where $r_{CS,F}$ is the same as above and PR_F is the rate of production of blood-borne F.

Discussion

It is more than 20 years since Bloch (1945) demonstrated that circulating cholesterol may be converted *in vivo* to pregnanediol. More recently, several other investigators have corroborated the finding that this sterol may be used by human subjects as an intermediate in the formation of steroid hormones. Thus, Ungar and Dorfman (1953) were able to demonstrate a conversion of orally administered [4-^{14}C]cholesterol to urinary androsterone and etiocholanolone. Werbin and LeRoy (1954), after the intravenous administration of labeled cholesterol to a human, isolated labeled tetrahydrocortisone from urine. Saroff *et al.* (1963), found that urine collected for 80 days following the injection of [4-^{14}C]cholesterol into a patient having an adrenal carcinoma, contained 7% of the label in a variety of C_{19} and C_{21} metabolites. That cholesterol may be converted to the estrogenic hormones was also established by Werbin *et al.* (1957) when they demonstrated the conversion of the labeled sterol to urinary estrone.

The experiments described here have been designed to assess the role of blood-borne cholesterol sulfate in the formation of the two most abundant adrenal secretory products, dehydroisoandrosterone sulfate and cortisol. It has already been shown (Roberts *et al.*, 1964) that CS, when introduced into the blood supplying an adrenal tumor, may be converted to a variety of 3β-sulfoxy Δ⁵-steroid metabolites. Its presence in human plasma made it conceivable that the sulfated sterol transported in the general circulation, could serve as a precursor of DS, cortisol, and perhaps other adrenal secretory products. The significance of circulating CS as a precursor was evaluated from the rate at which CS is introduced, *de novo*, into the blood (production rate in blood, PR_{CS}) and the fraction of PR_{CS} which is converted to steroids (the ρ values). The high rate of production observed is of special interest in view of the fact that the immediate precursor of CS is still unknown. Moreover, it has not been possible to demonstrate the conversion of cholesterol to CS using homogenates of bovine adrenals, bovine liver, and a human adrenal tumor (K. D. Roberts, unpublished data).

From the results of the studies described here it may be concluded that the major metabolic transformation of circulating CS is its conversion to cholesterol. This conclusion follows from the results obtained from subject 2, where the free cholesterol and the esterified cholesterol isolated from the plasma sample taken 74 hr following the injection of the tracer, were found to have specific activities of 280 and 50 dpm/mg, respectively. The concentration of free cholesterol in this subject was 540 mg/l. of plasma while that of esterified cholesterol was 1950 mg/l. Assuming that 25 g is a minimum value for the miscible pool of cholesterol (see below), it can be estimated that more than one-half of the labeled CS tracer injected had been converted to free or esterified cholesterol (2.6×10^6 from 4.8×10^6 dpm injected). There is reason to believe that cholesterol sulfate was converted to cholesterol to an extent larger than that indicated by these figures. For instance, reports in the literature have suggested that the value for the miscible pool of cholesterol is much larger than that assumed above. Chobanian *et al.* (1962) calculated a "miscible pool of cholesterol" to be about 200 g. They obtained this figure by extrapolation to zero time of the linear plot on a semilogarithmic scale of the slow component of the plasma-free cholesterol specific activity curve. Although such a procedure obviously leads to a gross overestimation, examination of the curves presented by these authors indicates the existence of more than one pool of distribution of plasma cholesterol. The value of 25 g assigned to the miscible pool in the above calculation was arrived at by analysis of specific activity curves kindly provided by Dr. DeWitt Goodman and is similar to the value which would result from extrapolating the early part of the cholesterol specific activity curves shown in the above-mentioned reference (Chobanian *et al.*, 1962) to zero time. The 2.6×10^6 dpm of [^{14}C]cholesterol (both free and esterified) estimated to be present in the miscible pool at 74 hr after injection of the tracer, does not include labeled cholesterol present in other pools nor does it include labeled cholesterol metabolized during the first 3 days of the experiment.

It may also be concluded from the present experiments that circulating cholesterol sulfate does not contribute significantly to the production of adrenal steroids in these subjects. The extent to which cholesterol sulfate was converted to cortisol, dehydroisoandrosterone, or dehydroisoandrosterone sulfate was determined from calculations using the specific activities of metabolites isolated from urine collected for 9 days following injection of the tracer. This extent of conversion was found to account for only a negligible fraction of the rate of production of these steroids. Even if a larger conversion of the cholesterol sulfate tracer to steroids were detected by extending the collection time of the urine, the slow appearance of the isotope in the metabolites would suggest peripheral conversion of cholesterol sulfate to cholesterol prior to the conversion of cholesterol sulfate to the secretory products and would not indicate direct utilization of blood-borne cholesterol sulfate for steroidogenesis. This conclusion follows from the observation (Roberts *et al.*, 1964) that, after the administration of labeled cholesterol sulfate into the splenic artery of a patient with an adrenocortical carcinoma, the appearance of radioactivity in urinary metabolites was rapid (0.7, 0.5, and 0.2% of the injected dose as urinary dehydroisoandrosterone sulfate during days 1–3, respectively). Since the half-life of circulating cholesterol sulfate is about 15 hr, a more rapid appearance of radioactivity in urine would have been expected in our present study if plasma cholesterol sulfate, *per se*, were an important intermediate in adrenal steroidogenesis.

The suggestion of Roberts *et al.* (1964) that cholesterol sulfate is an important intermediate deserves reexamination since the results described in this paper indicate that cholesterol sulfate circulating in blood is an insignificant precursor of the adrenal secretory product, dehydroisoandrosterone sulfate. The results of Krum *et al.* (1964) have already indicated that plasma cholesterol is the principal precursor of the adrenal hormone, cortisol. Krum and his co-workers fed cholesterol to dogs for several weeks until the plasma cholesterol had reached a constant specific activity. Cortisol, isolated from plasma at this time, was found to have a specific activity equal to that of cholesterol, thus indicating that this sterol was the sole precursor of the hormone. Analogous results were obtained in guinea pigs by Werbin and Chaikoff (1961). If these conclusions can be extrapolated to humans, then cholesterol sulfate could be an important intermediate of cortisol only if circulating cholesterol were sulfated in the adrenals. The possibility of peripheral sulfation of cholesterol and subsequent utilization of circulating cholesterol sulfate has been eliminated by the present experiments.

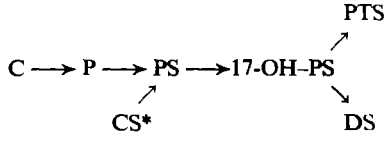
On the other hand, the work of Krum *et al.* as well as that of Werbin and Chaikoff, did not necessarily bear on the progenitors of dehydroisoandrosterone sulfate. It is conceivable that adrenal cortisol and other Δ^4-3-keto steroids are derived from plasma cholesterol but that dehydroisoandrosterone sulfate is not.

The possibility that dehydroisoandrosterone sulfate arises in the adrenal from cholesterol sulfate which, in turn, is derived from blood-borne cholesterol by sulfation *in the adrenals* also appears to be unlikely from the available evidence. *In vitro* experiments using either minces or homogenates of bovine adrenals and of human adrenal carcinomas, have all failed to effect the sulfation of cholesterol in spite of the fact that many other hydroxylated steroids, among them dehydroisoandrosterone, pregnenolone, desoxycorticosterone, and corticosterone have all been shown to serve as substrates for adrenal sulfokinase. However, the actual significance of these failures is questionable. For example, the high concentrations of cholesterol present in adrenal tissue may dilute the radioactive cholesterol used in these experiments to such an extent that a negligible number of counts would appear in the product of sulfation. Moreover, it can be esti-

mated that about 30 g of free cholesterol and 100 g of esterified cholesterol are presented daily to the human adrenals. Obviously, even if the percentage conversion of blood cholesterol to cholesterol sulfate in the adrenal is small, the mass of sterol sulfate synthesized from this huge source could be a substantial quantity.

The results of Roberts *et al.* (1964), were taken as evidence that cholesterol sulfate was an important intermediary in the formation of 3β-hydroxy Δ^5-steroids. These workers found that when doubly labeled cholesterol sulfate was introduced into an adrenal cortical tumor, the urinary steroid sulfates, pregnenetriol sulfate, and dehydroisoandrosterone sulfate isolated from the patient's urine, collected 24 hr following the injection of the tracer, possessed practically identical specific activities with respect to tritium and ^{35}S. Moreover, the $^3H/^{35}S$ ratios in these two metabolites were very similar to the ratio present in the labeled cholesterol sulfate administered. These findings were those that would be expected if the administered tracer were converted to urinary sulfates without cleavage of the sulfate group. Consequently, these results led to the conclusion that the urinary sulfates, dehydroisoandrosterone sulfate, and pregnenetriol sulfate, have common sulfated intermediates. Since the contribution of 3β-hydroxy Δ^5-intermediates to the urinary sulfates appeared to be small, it was further concluded that the isolated C-21 and C-19 steroidal sulfates are synthesized predominantly through sulfated intermediates using cholesterol sulfate as a precursor. Additional support for this idea was considered to be provided by the subsequent isolation of cholesterol sulfate from bovine adrenals (Drayer *et al.*, 1964; Drayer and Lieberman, 1965) and finally, from two adrenal tumors removed from patients with Cushing's syndrome (Dr. N. M. Drayer, unpublished results).

However, it is possible to interpret the results of Roberts *et al.* (1964) by means that do not require a large production of cholesterol sulfate by the carcinoma. For example, it is conceivable that both dehydroisoandrosterone sulfate and pregnenetriol sulfate have, as a common intermediate, pregnenolone sulfate, which is endogenously produced from pregnenolone by sulfation in the tumor. If the labeled cholesterol sulfate were metabolized to pregnenolone sulfate in the adrenals without loss of the [^{35}S]sulfate group, the $^3H/^{35}S$ ratios in the urinary dehydroisoandrosterone sulfate and pregnenetriol sulfate could be equal since both are derived from a common intermediate, pregnenolone sulfate. Since pregnenolone may be considered to be derived from cholesterol, the following scheme illustrates how the radioactive cholesterol sulfate (CS*) could label pregnenolone sulfate, dehydroisoandrosterone sulfate, and pregnenetriol sulfate (PTS) even though no unlabeled cholesterol sulfate were produced endogenously.



From the above analysis, it is clear that evidence for the intermediacy of cholesterol sulfate in the formation of the adrenal secretory product, dehydroisoandrosterone sulfate, is equivocal. The data presented in the present paper prove that blood-borne cholesterol sulfate, in the normal subject studied, is not an important precursor of dehydroisoandrosterone sulfate. Whether this would be true in a patient having a virilizing tumor has not been tested. More importantly, however, it must be recognized that the results described in this paper have no bearing on the role which cholesterol sulfate, produced *in the adrenals* (or in other steroid-producing glands), plays in the formation of 3β-hydroxy Δ^5-steroids. The results reported here have not excluded the possibility that intracellular cholesterol sulfate, formed in the adrenal and not in equilibrium with circulating cholesterol sulfate, serves as an intermediate of dehydroisoandrosterone sulfate in the adrenal.

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Added in Proof

Although several other investigators have failed to demonstrate the sulfation of cholesterol *in vitro* (Nose and Lipmann, 1958; Keller and Blennemann, 1961; Concolino and Ladany, 1966, unpublished results obtained in this laboratory using the supernatant fraction of guinea pig adrenals), Banerjee and Roy (1966) have recently reported that the sulfotransferases present in guinea pig liver can effect this conversion.

References

Banerjee, R. K., and Roy, A. B. (1966), *Mol. Pharmacol. 2*, 56.

Baulieu, E. E. (1963), *J. Clin. Endocrinol. Metab. 22*, 501.

Bloch, K. (1945), *J. Biol. Chem. 157*, 661.

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

Chobanian, A. V., Burrows, B. A., and Hollander, W. (1962), *J. Clin. Invest. 41*, 1738.

Cook, R. P., and Rattray, J. B. M. (1958), *in* Cholesterol, Cook, R. P., Ed., New York, N. Y., Academic, p 117.

Crepy, O., and Rulleau-Meslin, F. (1960), *Rev. Franc. Études Clin. Biol. 5*, 283.

Drayer, N. M., Roberts, K. D., Bandy, L., and Lieberman, S. (1964), *J. Biol. Chem. 239*, PC3112.

Drayer, N. M., and Lieberman, S. (1965), *Biochem. Biophys. Res. Commun. 18*, 126.

Gurpide, E., Mann, J., and Lieberman, S. (1963), *J. Clin. Endocrinol. Metab. 23*, 1155.

Keller, H., and Blennemann, H. (1961), *Z. Physiol. Chem. 324*, 125.
Krum, A. A., Morris, M. D., and Bennett, L. L. (1964), *Endocrinology 74*, 543.
Martin, R. P. (1957), *Biochem. Biophys. Acta 25*, 408.
McKenna, J., and Norymberski, J. K. (1960), *Biochem. J. 76*, 60 P.
Moser, H. W., Moser, A. B., and Orr, J. C. (1966), *Biochem. Biophys. Acta 116*, 146.
Nose, Y., and Lipmann, F. (1958), *J. Biol. Chem. 233*, 1348.
Porter, C. C., and Silber, R. H. (1950), *J. Biol. Chem. 185*, 201.
Raggatt, P. R., Dean, P. D. G., and Whitehouse, M. W. (1965), *Proc. Biochem. Soc. 96*, 26P.
Roberts, K. D., Bandy, L., Calvin, H. I., Drucker, W. D., and Lieberman, S. (1964), *Biochemistry 3*, 1983.
Roginsky, M. S., and Schick, M. (1966) *Arch. Internal Med. 117*, 673.
Roy, A. B. (1956), *Biochem. J. 62*, 41.
Sandberg, E., Gurpide, E., and Lieberman, S. (1964), *Biochemistry 3*, 1256.
Saroff, J., Slaunwhite, W. R., Costa, G., and Sandberg, A. A. (1963), *J. Clin. Endocrinol. Metab. 23*, 629.
Siiteri, P. K. (1963), *Steroids 2*, 687.
Tait, J. F. (1963), *J. Clin. Endocrinol. Metab. 23*, 1285.
Ungar, F., and Dorfman, R. I. (1953), *J. Biol. Chem. 205*, 125.
Werbin, H., Bergenstal, D. M., Gould, R. G., and Le Roy, G. V. (1957), *J. Clin. Endocrinol. Metab. 17*, 337.
Werbin, H., and Chaikoff, I. L. (1961), *Arch. Biochem. Biophys. 93*, 476.
Werbin, H., and LeRoy, G. V. (1954), *J. Am. Chem. Soc. 76*, 5260.
Wusteman, F. S., Dodgson, K. S., Lloyd, A. G., Rose, F. A., and Tudball, N. (1964), *J. Chromatog. 16*, 334.

Peptides of L- and D-Alanine. Synthesis and Optical Rotations*

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ABSTRACT: Peptides (35) of alanine (up to hexapeptides) containing L and D residues at predetermined positions were synthesized by condensing benzyloxycarbonyl-alanine with the appropriate intermediate peptide *p*-nitrobenzyl esters. Separation of alanine peptides up to the dodecapeptide was achieved by high-voltage electrophoresis at pH 1.4. Purity and retention of optical configuration were better than 99.5% as checked by paper electrophoresis, by the action of exopeptidases, and by end-group analysis. The specific rotations of the free as well as of the blocked peptides were determined at a number of wavelengths. It was found that molar rotations could be expressed to a good approximation as the sum of three different "molar residue rotations": one each for the amino-terminal, nonterminal, and carboxyl-terminal residue, taking values of opposite sign for L and D residues [E. Brand and B. F. Erlanger (1950), *J. Am. Chem. Soc. 73*, 3314]. The possibility of assigning additive rotational parameters to the various types of peptide bond chromophors was investigated.

It was found that out of the six necessary parameters only one, the rotational contribution of an internal peptide bond joining two L residues (or two D residues), can be evaluated unequivocally. If an assumption is made about one of the other "chromophor rotations," the remaining four can be computed. It is shown that this treatment is equivalent to the Brand–Erlanger treatment when extended to five different "molar residue rotations."

In order to provide suitable compounds for a study of the stereospecificity of proteolytic enzymes a series of alanine peptides composed of L- and D-alanyl residues in predetermined positions was synthesized. Altogether 35 peptides, from dialanine to hexaalanine, were prepared. A number of these have already been reported in the literature (Erlanger and Brand, 1951; Brand *et al.*, 1951, 1952). The availability of this series of alanine peptides made it possible to carry out a systematic comparison of their optical rotatory properties and the "residue rotation" of an L-alanine residue in a random poly-L-alanine chain could be evaluated.

Materials and Methods

Reagents and Solvents. Benzyloxycarbonyl-L- (and D-) alanine was prepared according to Bergmann and Zervas (1932). Isobutyl chloroformate (Eastman Kodak) and *p*-nitrobenzyl chloride (Fluka) were used

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