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Regulation of Rat Testis Steroid Sulfatase. A Kinetic Study*

Albert D. Notation and Frank Ungar

ABSTRACT: A variety of unconjugated steroids were found to inhibit the *in vitro* cleavage of dehydroepiandrosterone sulfate by rat testis tissue. The glucosiduronates of cortisol and cortisone had virtually no effect on the cleavage of dehydroepiandrosterone sulfate whereas the liberation of labeled dehydroepiandrosterone was efficiently suppressed in the presence of pregnenolone sulfate. The emerging trend of effective inhibition involves testosterone, estradiol, and possible biochemical precursors including pregnenolone. The inhibition constants of dehydroepiandrosterone ($K_i = 7.5 \times 10^{-6}$ M) and of testosterone ($K_i = 7.0 \times 10^{-6}$ M) for dehydroepiandrosterone sulfate cleavage ($K_m = 1 \times 10^{-5}$ M) were comparable with the inhibition constants of pregnenolone ($K_i = 8 \times 10^{-6}$ M) and of testosterone ($K_i = 8 \times 10^{-6}$ M) for pregnenolone sulfate cleavage ($K_m = 1 \times 10^{-5}$ M). Cholesteryl sulfate cleavage ($K_m = 5 \times 10^{-5}$ M) was inhibited by cholesterol ($K_i = 12 \times 10^{-6}$ M), testosterone ($K_i = 7.0 \times 10^{-6}$ M), and pregnenolone sulfate ($K_i = 4 \times 10^{-6}$ M). The kinetic data in all cases are compatible with competitive inhibition. Whatever the significance of the role of steroid sulfates in steroid hormone production might be, the present studies indicate that an effective control exists for the regulation of hormone synthesis *via* this pathway.

An initial report described how either exogenous dehydroepiandrosterone[1] or testosterone could inhibit the *in vitro* cleavage of DHEA-SO_4 in rat testis tissue. In contrast to this, the conversion of DHEA into its sulfate appeared to be independent of the amount of DHEA-SO_4 present. In spite of the low interconversion of DHEA and its sulfate, this inhibitory effect provides some basis for a possible regulation of the sulfate contribution to steroid sex hormone biosynthesis (Notation and Ungar, 1968). This report describes other steroid compounds which can inhibit the *in vitro* cleavage of DHEA-SO_4. Kinetic comparisons of the cleavages and selected inhibitions of the cleavages *in vitro* of DHEA-SO_4 pregnenolone sulfate, and cholesteryl sulfate, respectively, are also presented.

Methods and Materials

Dehydroepiandrosterone-7α-^{3}H sulfate, ammonium salt (labeled DHEA-SO_4) (15 Ci/mmole), pregnenolone-7α-^{3}H sulfate, ammonium salt (labeled-Preg-SO_4) (12.2 Ci/mmole), and cholesteryl-7α-^{3}H sulfate, ammonium salt (5.2 Ci/mmole) were obtained from the New England Nuclear Corp., Boston. Unlabeled unconjugated steroids were obtained from Mann Research Laboratories. Unlabeled DHEA-SO_4-NH_4, Preg-SO_4-NH_4, and cholesteryl-SO_4-NH_4 were synthesized, solvents were prepared, and steroid purity was checked by methods previously described (Notation and Ungar, 1968). Cortisol-21-glucosiduronic acid and cortisone-21-glycosiduronic acid were obtained from Dr. V. X. Mattox.

Preparation of Rat Testis Tissue Homogenate. Sprague–Dawley rats (180–200 g) were treated with human chorionic gonadotrophin for 3 days prior to sacrifice and homogenate of testis tissue was prepared as previously reported (Notation and Ungar, 1968). The buffer used in these latter experiments was a Krebs–Ringer 0.1 M bicarbonate buffer (pH 7.4) containing 200 mg of glucose/100 ml and 10 mg of fumaric acid/100 ml. Whole homogenates were used initially in the study of DHEA-SO_4 cleavage and subsequently compared with several particulate fractions which were initially obtained by removing the heavy tissue fragments from whole homogenates centrifuged at 4000*g* for 10 min.

Incubation and Extraction Procedures. Steroid substrates were dissolved in 10 μl of propylene glycol and the flasks (22-ml screw-top vials with foil-lined caps) were cooled on ice. Cold testis tissue homogenate (1.0 ml containing 100 mg of tissue) and a cofactor solution (50 μl) containing 100 μg each of ATP, β-DPN, and K_2SO_4 were added to each flask. Incubations were carried out in duplicate at 37° under an atmosphere of O_2–CO_2 (95:5) in a Dubnoff metabolic shaker. The incu-

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[1] The following trivial names are used: dehydroepiandrostrone (DHEA), 3β-hydroxy-5-androsten-17-one; pregnenolone (Preg), 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; testosterone, 17β-hydroxy-4-androsten-3-one; androsterone, 3α-hydroxy-5α-androstan-17-one; etiocholanolone, 3α-hydroxy-5β-androstan-17-one; estradiol, estra-1,3,5(10)-triene-3,17β-diol; cortisol, 11β,17α,21-hydroxy-4-pregnene-3,20-dione; cortisone, 17α,21-dihydroxy-4-pregnene-3,11,20-trione.

TABLE I: Assay Efficiency: Per Cent Recoveries of Steroid in the Toluene Phase.[a]

Steroid (M)	From Tissue	From Buffer Blanks: Toluene Extraction of Buffer	From Buffer Blanks: Buffer Extraction of Toluene
Dehydroepiandrosterone ($0.030–1.7 \times 10^{-4}$)	88	92	93
Pregnenolone ($0.030–1.7 \times 10^{-4}$)	96	100	100
Cholesterol ($0.030–1.7 \times 10^{-5}$)	33	51	69
Cholesterol ($1.7 \times 10^{-5}–1.7 \times 10^{-4}$)	25	51	70

[a] Over-all recoveries of varying concentration of DHEA-7α-^{3}H, Preg-7α-^{3}H, and cholesterol-7α-^{3}H in toluene from tissue preparations, from buffer blanks, and from preparations in which the labeled compounds were dissolved in the toluene phase, then partitioned with aqueous buffer. Determinations were carried out in duplicate and summarized as shown.

TABLE II: The Inhibitory Effects of Various Steroid Compounds on the *in Vitro* Cleavage of Tracer DHEA-SO_4 by Rat Testis Tissue.[a]

	Per Cent[b] Inhibition of DHEA-7α-^{3}H-SO_4 Cleavage				
	Inhibitor Concn (moles/l.)				
	3.47×10^{-6}	1.73×10^{-5}	3.47×10^{-5}	3.47×10^{-4}	1×10^{-1}
Pregnenolone	18	67	87		
Progesterone	13	67	84		
17α-OH-Pregnenolone		42	66		
17α-OH-Progesterone	33	68	79		
5-Androstene-3β,17β-diol	39	80	72		
Dehydroepiandrosterone	25	59	85		
4-Androstene-3,17-dione	17	54	87		
5-Pregnene-3β,20α-diol	41	80			
Estradiol	21		85		
Testosterone	27	66	90		
Androsterone			60		
Etiocholanolone			64		
Cholesterol			52	98	
Cortisol			50	66	
Cortisol glucuronide			29		
Cortisone	7	20			
Cortisone glucuronide	0	5			
Pregnenolone sulfate			94	97	
Phosphate ion					22[c]

[a] Incubations in duplicate were carried out for 15 min using 0.1 μg of substrate in the presence of varying concentrations of test agents as shown. [b] Each value presented is the average of duplicate samples. The substrate concentration was 0.1 μg/ml. [c] At 4 μg/ml of substrate the inhibition is 34%.

bations were terminated at the desired time points by cooling each flask in an ice bath and immediately adding 3.0 ml of 0.1 M NaOH solution. The liberated unconjugated steroid-^{3}H was determined following a partition between 15 ml of toluene (containing scintillation indicators) and the alkaline incubation mixture according to the procedures of Burstein and Dorfman (1963). Particular care had to be exercised in the procedure in which the aqueous phase, solidified by freezing in a Dry-Ice–methanol bath, was separated from the toluene phase by filtration.

Radioactive Counting. Scintillation fluid consisted of 0.5% of 2,5-diphenyloxazole and 0.05% of 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene. Extracts of this solvent were assayed for radioactivity in a Packard Tri-Carb scintillation spectrophotometer, Model 314 XE.

Results

The efficiency of the extraction procedure was checked using cholesterol-7α-^{3}H, DHEA-7α-^{3}H, and pregnenolone-7α-^{3}H in separate determinations. Steroids-^{3}H

in the concentration used in the experiments were dissolved in 10 μl of propylene glycol, suspended in 4 ml of premixed alkaline solution containing either 1 ml of buffer or 1 ml of tissue preparation, then extracted with 15 ml of toluene scintillation fluid. Another series of labeled steroids in propylene glycol was first dissolved in the toluene phase and then extracted with aqueous buffer solution. The per cent recoveries are tabulated in Table I.

Experimental blanks consisting only of labeled tracer in buffer were determined for each radioactive substrate concentration and used as the background reading for their respective series. All determinations were carried out in duplicate and the average of the duplicate readings was used as the experimental reading. In most instances, the duplicate values were well within $\pm 4\%$ of the average value shown. K_m and K_i values were calculated by the methods described by Dixon (1953).

A series of steroid compounds were screened for inhibitory actions on the *in vitro* cleavage of labeled DHEA-SO_4 at 15 min. The results are presented as per cent inhibition (Table II). The most effective inhibitions were observed for testosterone, estradiol, 4-androstene-3,17-dione, DHEA, 5-androstene-3β,17β-diol, pregnenolone, progesterone, and pregnenolone sulfate. Etiocholanolone and androsterone were less efficient. Cholesterol, cortisol, and cortisone exhibit comparatively low inhibition while the glucosiduronates of cortisol and cortisone appeared to be virtually ineffective.

A K_m value of 1×10^{-5} M was obtained for the cleavage of labeled DHEA-SO_4 by whole homogenates of rat testis tissue incubated for 15 min. No significant change in K_m value was observed when a resuspended microsomal fraction from rat testis tissue of the same concentration was used.

Graphical data for the K_m (Figure 1), the K_i for product inhibition (Figure 2), and the K_i for testosterone inhibition (Figure 3) of DHEA-SO_4 cleavage are presented as typical examples in this study. A list of K_m and K_i values obtained are presented in Table III. The suppression of cholesteryl sulfated cleavage by pregnenolone sulfate ($K_i = 4 \times 10^{-6}$ M) is represented in Figure 4. The cleavage of Preg-SO_4 in the presence of varying concentrations of DHEA-SO_4 appeared to remain unchanged.

A test for the reversibility of the testosterone inhi-

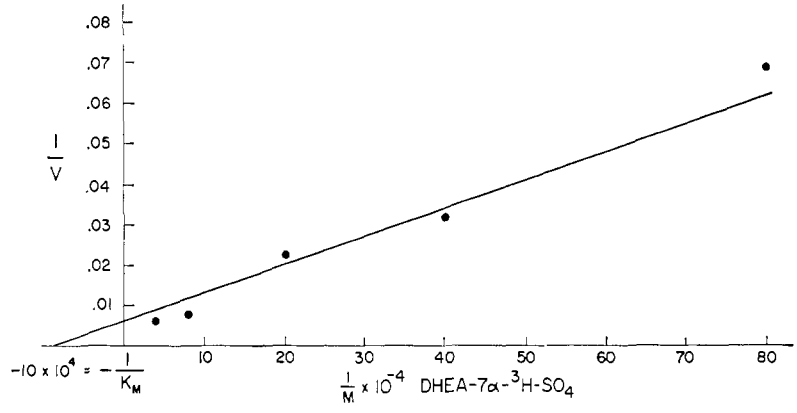

FIGURE 1: A K_m value of 1×10^{-5} M was obtained for the cleavage of DHEA-SO_4 *in vitro* by rat testis tissue. Incubations in duplicate were carried out for 15 min and the average of each pair of readings was plotted as a single point.

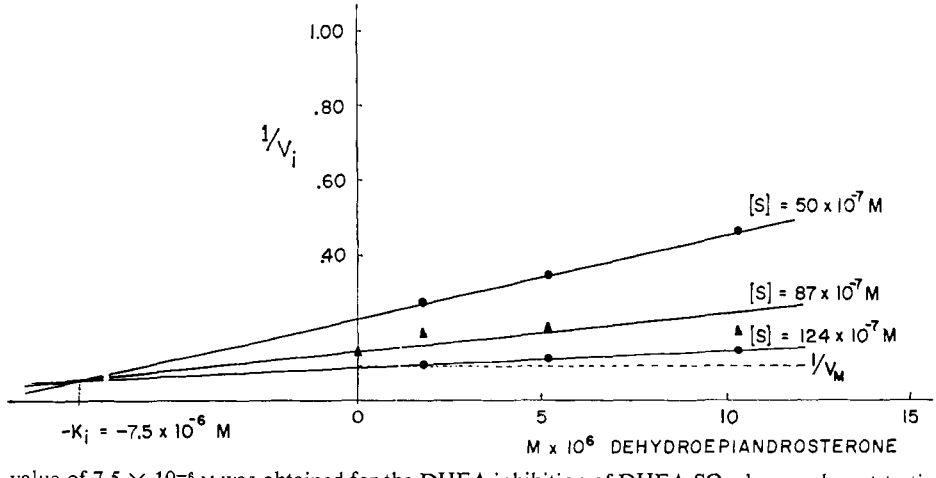

FIGURE 2: A K_i value of 7.5×10^{-6} M was obtained for the DHEA inhibition of DHEA-SO_4 cleavage by rat testis tissue. Varying inhibition concentrations in duplicate were tested at each of three substrate concentrations at the 5-min time point and the average of each pair of readings was plotted as a single point. The kinetic data are compatible with competitive inhibition.

bition of DHEA-SO_4 cleavage was conducted in the following manner. Exogenous testosterone (15.6×10^{-6} and 20.8×10^{-6} M, respectively) was added to aliquots of whole homogenate of rat testis tissue cooled in an ice bath. After thorough mixing, the homogenates were centrifuged at 32,000g for 40 min at 4°. The supernatant was drawn off and the remaining precipitates were suspended in fresh Krebs–Ringer bicarbonate buffer in

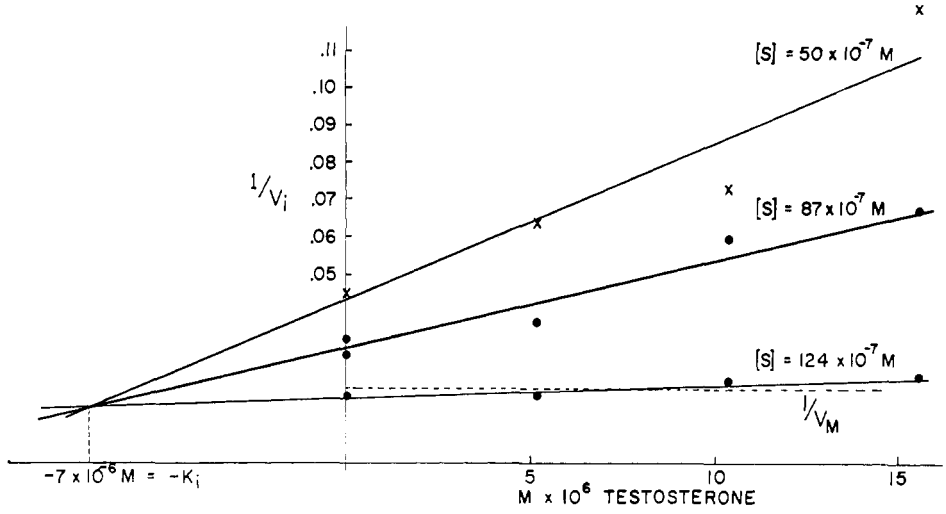

FIGURE 3: A K_i value of 7×10^{-6} M was obtained for the testosterone inhibition of DHEA-SO_4 cleavage by rat testis tissue. Varying inhibitor concentrations in duplicate were tested at each of three substrate concentrations at the 15-min time point and the average of each pair of readings was plotted as a single point. The kinetic data are compatible with competitive inhibition.

TABLE III: A Summary of the Kinetic Data on the Cleavage Reported as K_m Values of DHEA-SO_4, Preg-SO_4, and Cholesteryl Sulfate, and Their Respective K_i Values for Product Inhibition and Testosterone Inhibition.[a]

Sulfate Substrate	K_m (M)	Inhibitor Constant, K_i	
		Product	Testosterone
Dehydroepiandrosterone	1×10^{-5}	7.5×10^{-6}[b]	7×10^{-6}
Pregnenolone	1×10^{-5}	8×10^{-6}	8×10^{-6}
Cholesterol	5×10^{-5}	12×10^{-6}	7×10^{-6}

[a] Each K_i value was determined from a series of inhibitor concentrations determined at three different substrate concentrations and expressed in a graphical extrapolation. [b] Determined at the 5-min time point.

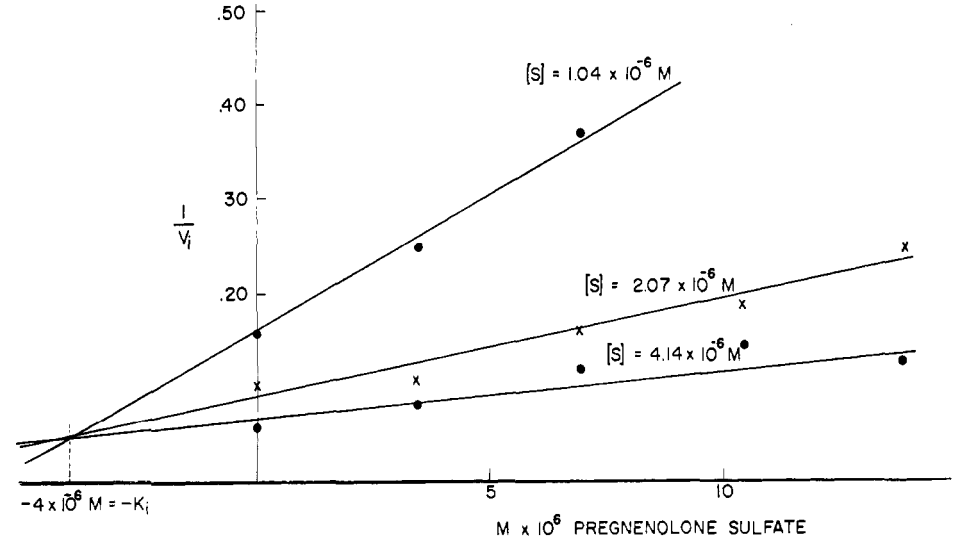

FIGURE 4: A K_i value of 4×10^{-6} M was obtained for the Preg-SO_4 inhibition of cholesteryl sulfate cleavage by rat testis tissue. Varying inhibition concentrations in duplicate were tested for each of three substrate concentrations at the 15-min time point and the average of each pair of readings was plotted as a single point. The kinetic data are compatible with kinetic inhibition.

a volume equal to that of the discarded supernatant. There was no evidence of testosterone inhibition when these treated fractions were used to cleave DHEA-SO_4.

Discussion

The testosterone and dehydroepiandrosterone inhibitions of DHEA-SO_4 cleavage by rat testis tissue homogenates assayed by Celite 545 column chromatography initially reported (Notation and Ungar, 1968) were verified by the extraction assay procedures described by Burstein and Dorfman (1963). Separations of unconjugated steroid by a single extraction with toluene was found to give a consistent recovery of 90% over a wide range of concentrations of pregnenolone and DHEA. The toluene extraction of cholesterol from tissue preparations was surprisingly low at 25%. The unfavorable partition of cholesterol in this system, particularly in the presence of tissue, is noteworthy but did not appear to be detrimental to the assay in this study since there was a consistent level of extraction over the range of concentrations employed.

In an initial survey pregnenolone and a number of its related unconjugated steroids including testosterone and estradiol were all found to be effective inhibitors of DHEA-SO_4 cleavage. The inhibition by cholesterol was observed to be of a lower order similar to that of cortisol and cortisone whereas the glucosiduronates of cortisol and cortisone were virtually ineffective. The reduced ring A metabolites etiocholanolone and androsterone also were less effective inhibitors than testosterone. The emerging trend of inhibition involves a number of steroids that includes the sex hormones and their unconjugated precursors as distantly related as pregnenolone.

Phosphate ion inhibits sulfatase activity (Roy, 1957) but the per cent inhibition was observed to be relatively low in this study. The inhibition observed for 10^{-1} M phosphate ion is comparable with the inhibition caused by 3.5×10^{-6} M steroid. Moreover, DHEA-SO_4 cleavage inhibition by DHEA and by testosterone have been observed in 10^{-1} M phosphate ion. It is difficult to conceive any situation in which phosphate ion may regulate and/or greatly interfere with any steroid regulation of DHEA-SO_4 cleavage.

Rat testis steroid sulfatase enzyme was described (Burstein and Dorfman, 1963) in terms of a number of kinetic criteria including a K_m value of 1.2×10^{-5} M DHEA-SO_4 which we were able to verify. A more elaborate characterization of the rat testis steroid sulfatase system was carried out using their kinetic assay procedures. Studied individually, the cleavage of DHEA-SO_4 ($K_m = 1 \times 10^{-5}$ M) appeared to be identical with the cleavage of pregnenolone sulfate ($K_m = 1 \times 10^{-5}$ M) but more efficient than the cleavage of cholesteryl sulfate ($K_m = 5 \times 10^{-5}$ M). Product inhibition studies followed the same trend. DHEA-SO_4 cleavage inhibited by DHEA ($K_i = 7.5 \times 10^{-6}$ M) was comparable with Preg-SO_4 cleavage inhibited by pregnenolone ($K_i = 8 \times 10^{-6}$ M) but probably different from cholesteryl sulfate cleavage inhibited by cholesterol ($K_i = 12 \times 10^{-6}$ M). The K_i determination for DHEA was carried out at the 5-min time point because DHEA in low concentrations appeared to be metabolized too rapidly to give accuracy over a 15-min time period, and the results using higher concentrations of DHEA were less reproducible. Although the additions of DPN to these incubations caused oxidation of DHEA this cofactor was maintained as one of the standard conditions used throughout this comparative study. In this way it was thought to reduce interference caused by product inhibition in the other K_m and K_i determinations. The cleavage of cholesteryl sulfate was suppressed in the presence of Preg-SO_4 ($K_i = 4 \times 10^{-6}$ M). There is some evidence indicating that pregnenolone sulfate also suppresses the cleavage of DHEA-SO_4 (Table II). The cleavage of Preg-SO_4 did not appear to change significantly in the presence of varying amounts of DHEA-SO_4. The apparent substrate specificities, the demonstrable substrate competition, and the constant K_i values obtained for the testosterone inhibition using different substrates are compatible with the suggestion that a single steroid sulfatase enzyme is being observed in this system. The enzyme specificity for steroid-3β-yl sulfates of the 5α and Δ^5 series as reported by Roy (1957) should now be extended, however, to include increased specificity dependent upon the substituents at the C-17 position since pregnenolone sulfate appears to be a more acceptable substrate than DHEA-SO_4 which in turn is better than cholesteryl sulfate. As a working hypothesis one may regard the steroid inhibitions of steroid sulfate cleavage to be reversible and competitive; however the mechanism of these inhibitions has yet to be elucidated.

An interpretation of the kinetic data of this study may be made as follows. It is likely that once cholesteryl sulfate is formed in the testis it is not readily cleaved, but may be metabolized with its sulfate moiety intact to some other product which is more labile to enzymatic cleavage. DHEA-SO_4 by itself appears to be cleaved equally as fast as Preg-SO_4, except that in combination, Preg-SO_4 and perhaps some other sulfates are the preferred substrates. The 3β-yl sulfates of 20-hydroxycholesterol, 17β-hydroxypregnenolone, and 5-androstene-3β,17β-diol cannot be excluded as possible participants at this stage of the investigation. If the plasma DHEA-SO_4 is available to gonadal tissue, then such a subordinate substrate role is eminently suited to function as a precursor reservoir in steroid sex hormone production. Other steroid sulfates have not been observed to accumulate in the plasma possibly because they could be more metabolically active than DHEA-SO_4. If this is the case, then the DHEA-SO_4 pool would be available to put out its maximum contribution during peak production of steroid sex hormones, and even more so if some other contributing route were blocked. Furthermore, it would always function as a hormonally dormant respository for storage and excretion. In that the accumulation of DHEA-SO_4 is apparently a continuing process, it is only necessary to maintain an upper concentration limit within the pool.

Aakvaag *et al.* (1964) reported that radioactive DHEA-SO_4 infused into the spermatic artery of a canine testis was converted *in vitro* into 4-androstene-3,17-dione and testosterone. They concluded that since the plasma levels of DHEA-SO_4 in humans are much higher than

those in dogs, plasma DHEA-SO_4 in the human might contribute significantly to steroid hormone production. Furthermore, Young and Hall (1968) observed the biosynthesis of cholesteryl sulfate by slices of rabbit testis. Based on these reports and other evidence steroid sulfates are present in gonadal tissue although their levels have not been documented.

Combinations of unconjugated steroids in low concentrations have not been tested for inhibitory effects on steroid sulfate cleavage but some kind of cumulative inhibition would be expected to occur. Furthermore, the concentrations and kinds of related steroid sulfates present will in part regulate the cleavage of any single steroid sulfate member. Clearly then, the contribution from steroid sulfates to steroid hormone production, although small, can be highly variable and well suited to be an auxiliary biosynthetic route.

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Synthesis of Cerebroside by Brain from Uridine Diphosphate Galactose and Ceramide Containing Hydroxy Fatty Acid*

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ABSTRACT: A crude microsomal fraction from the brains of young mice catalyzes the formation of galactosylceramide containing hydroxy fatty acids from 2-hydroxy fatty acid ceramide and uridine diphosphate galactose. The 2-hydroxy fatty acid ceramide is most effective as a substrate when it is spread over the large surface area offered by the diatomaceous earth, Celite, in the absence of detergent. The enzyme system exhibits high specificity: ceramide containing nonhydroxy fatty acids will not stimulate galactose incorporation and only uridine diphosphate galactose or a uridine diphosphate galactose generating system acts as a sugar donor. The product of the incubation mixture has been characterized as 2-hydroxy fatty acid galactosylceramide by a variety of chromatographic and chemical procedures. Some other properties of the system were investigated. The results are discussed and related to our present knowledge of sphingolipid metabolism.

An active psychosine[1]-synthesizing system has been demonstrated by Cleland and Kennedy (1960). Brady (1962) reported that psychosine was an acceptor for stearoyl-CoA in a rat brain microsomal system. On the basis of this work it has been generally accepted (Olson, 1966) that the biosynthetic route for cerebroside follows the route outlined in eq 1 and 2.

$$\text{sphingosine} + \text{UDPGal} \longrightarrow \text{pyschosine} + \text{UDP} \quad (1)$$

$$\text{psychosine} + \text{fatty acyl-CoA} \longrightarrow \text{cerebroside} + \text{CoA} \quad (2)$$

Since this scheme is at variance with a preliminary observation in this laboratory (Radin, 1959), that ceramide stimulates the incorporation of galactose into lipids, we have reinvestigated the biosynthesis of cerebroside.

Experimental Section

Materials.. UDPGal and dithiothreitol were purchased from Calbiochem (Los Angeles, Calif.). UDPGlc was a product of Sigma Chemical Co. (St. Louis, Mo.) and the nonionic detergent Tween 20 was obtained from Atlas Chemical Industries (Wilmington, Del.). Analytical grade Celite, a purified diatomaceous earth, was a Johns-Manville product. All other chemicals used were reagent grade and all solvents were redistilled before use. Radioactivity incorporation studies utilized uniformly labeled α-D-[^{14}C]glucose-1-P (10.3 mCi/mmole), uniformly labeled α-D-[^{14}C]galactose-1-P (5.0 mCi/mmole), and uniformly labeled [galactose-^{14}C]UDPGal (5.9 mCi/

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† Postdoctoral trainee, supported by Training Grant MH-07417 from the National Institute of Mental Health, U. S. Public Health Service.

[1] Abbreviations not listed in the 1966 issue of *Biochemistry 5*, 1445, are: nfa and hfa, nonhydroxy fatty acid and 2-hydroxy fatty acid (chain length may be indicated in a prefix, *e.g.*, C_{18}-nfa); LCB, a mixture of long-chain bases (sphingosine and some dihydrosphingosine); ceramide, fatty acylamide of LCB (the fatty acid may be indicated by a prefix); psychosine, LCB galactoside; cerebroside, galactosylceramide; BSC, beef spinal cord sphingopids.