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Cholesterol and Cholesterol Sulfate as Substrates for the Adrenal Side-Chain Cleavage Enzyme†

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ABSTRACT: The rates of the enzymatic cleavage of the side chains of cholesterol and cholesterol sulfate have been studied using subcellular fractions from the adrenal cortex of the rat and cow. Although impermeable to exogenous TPNH, intact mitochondria *in vitro* can convert doubly labeled [^{3}H]-cholesterol [^{35}S]sulfate into [^{3}H]pregnenolone [^{35}S]sulfate. When Ca^{2+} is present, exogenously added TPNH accelerates the rates of side-chain cleavage of both cholesterol sulfate and cholesterol. The addition of isocitrate also increases the oxidation of both substrates. However, succinate accelerates (by about 70%) the rate of side-chain cleavage of only cholesterol sulfate. The rate of oxidation of cholesterol is only slightly affected by succinate. Determination of the kinetic parameters of the two substrates indicated that the apparent K_m for cholesterol sulfate is smaller than that of cholesterol and also that the V_{max} for the conjugate is greater than that of the free sterol. Inhibition studies have shown that each of the substrates can inhibit the cleavage of the other. Cholesterol glucuronide inhibits the oxidation of neither cholesterol nor its sulfate.

One of the metabolic fates of cholesterol sulfate, a ubiquitous compound found in many mammalian tissues and body fluids, is its enzymatic conversion into pregnenolone sulfate[1] (Roberts *et al.*, 1967; Raggatt and Whitehouse, 1966; Young and Hall, 1969). The steroid conjugate serves as a substrate for the side-chain cleavage enzyme systems present in the adrenal and in the testes (Ponticorvo and Lieberman, unpublished results), through mechanisms that have been shown to occur with the retention of the sulfate moiety. In this regard, cholesterol sulfate is unique, since all other known esters of cholesterol are not transformed into C_{21} steroids without prior hydrolysis.

The experiments described in this paper were designed to investigate further the role of cholesterol sulfate as a substrate for the adrenal side-chain cleavage enzyme. Whether cholesterol sulfate can be converted into pregnenolone sulfate by

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[1] Abbreviations or trivial names used are: pregnenolone sulfate, 3β-hydroxypregn-5-en-20-one sulfate; pregnenolone, 3β-hydroxypregn-5-en-20-one; Et_3N, triethylammonium; progesterone, pregn-4-ene-3,20-dione; hbv, hold-back volume.

"intact" mitochondria and, if so, how its rate of conversion compares to that of cholesterol, are two of the problems that were studied. In addition, the ability of various agents to influence the rate of conversion of both substrates was examined. The rates of conversion and the apparent K_m's of the two substrates were also measured using, as a source of enzyme, acetone powders of adrenal mitochondria which supposedly are depleted of endogenous substrates. By making kinetic measurements with this preparation, the ability of either sterol to inhibit the side-chain cleavage of the other was investigated. To test for specificity, cholesterol glucuronide was also used as a potential inhibitor.

The results of the experiments reported in this paper indicate that cholesterol sulfate can be converted into its C_{21} product (pregnenolone sulfate) by intact mitochondria, and that its rate of cleavage and its K_m are comparable to those of free cholesterol. If these results have meaning for the conditions obtaining *in vivo*, cholesterol sulfate must be considered to be capable of serving as an efficient precursor of the steroid hormones, particularly those with which sulfate pathways have been implicated, *i.e.*, androgens, estrogens and, obviously, dehydroisoandrosterone sulfate.

Experimental Section

All tracers were purchased from New England Nuclear Corp. and were counted in a Packard TriCarb liquid scintillator spectrometer (Model 3375) in a toluene-based scintillation solution. Steroid sulfates were first dissolved in 2 ml of methanol before addition of 10 ml of the scintillation mixture. The ^3H and ^{35}S counts were corrected using the method described by Okita *et al.* (1957). Chromatography on Celite was performed essentially as described by Kelly *et al.* (1962). [1,2-^{3}H]Cholesterol (12.7 Ci/mmol) and [26-^{14}C]cholesterol (57.5 Ci/mol) were purified in system A, and were sulfurylated as previously reported (Roberts *et al.*, 1969). [^{35}S]Chlorosulfonic acid (3.64 Ci/mol) was converted into pyridine–sulfur trioxide, and was used to sulfurylate cholesterol (purified *via* the dibromide) as described by Roberts *et al.* (1969). The cholesterol [^{35}S]sulfate was purified by crystallization, whereas [^{3}H]cholesterol sulfate was purified on Celite in system B. Prior to their use as substrates, the tritiated and [^{35}S]cholesterol sulfates were combined and chromatographed in system B (Table I).

Each tracer steroid was purified by chromatography, and its radiochemical purity was established by diluting an aliquot of the tracer with a weighed quantity of carrier. The mixture was then recrystallized. Only when the specific activity of the purified material was, within experimental error, equal to the expected value, was the tracer considered to be devoid of contaminants and usable as a substrate. The tracer was always used within a few days of the purification process to avoid the accumulation of products of radiolysis.

The triethylammonium salt (Et_3N) of pregnenolone sulfate was synthesized from triethylamine sulfur trioxide by the method of Dusza *et al.* (1968). The substrate, [^{3}H]cholesterol, was diluted with purified carrier cholesterol to obtain a sample whose specific activity was approximately equal to that of the [^{3}H]cholesterol [^{35}S]sulfate.

Preparation of Mitochondria from Rat Adrenals. The procedure used was essentially that described by Sauer and Mulrow (1969). Twelve adult female rats were lightly anesthesized with ether and were decapitated. The adrenals were quickly removed, defatted, and were then kept on ice in solution A which was a 1% solution of bovine serum albumin containing sucrose (0.25 M), EDTA (1 mM), and Tris-HCl buffer (pH 7.4) (30 mM). The adrenals were added to 10 ml of solution A, and were homogenized in a Potter-Elvehjem homogenizer. The suspension was centrifuged for 10 min at 750*g*, yielding a pellet which was resuspended in 5 ml of solution A, homogenized, and recentrifuged at 750*g* for 10 min. The two supernatants were combined and centrifuged for 10 min at 6000*g*. The pellet thus obtained was resuspended in solution A, and centrifuged in this manner twice again. The mitrochondrial pellet from the final centrifugation was suspended in 1 ml of solution B which was a 1% solution of bovine serum albumin (fatty acid free, Pentex Corp.) containing sucrose (0.25 M), $MgCl_2$ (1 mM), and Tris-HCl buffer at pH 7.4 (30 mM). The protein concentrations of 50-μl aliquots of both solution B and the mitochondrial suspension were determined by the method of Lowry *et al.* (1951). The mitochondrial suspension was then diluted to 6 ml with solution B for use in the incubation experiments.

TABLE I: Celite Chromatography Systems.

(A) Methanol–1-propanol–water–toluene–isooctane (4:1:1.3:2:2) (reverse phase)
(B) Isooctane–ethyl acetate–*tert*-butyl alcohol–methanol–1 M NH_4OH (4.2:1.2:2:3)
(C) Isooctane–ethyl acetate, *tert*-butyl alcohol–methanol–1 M NH_4OH (2.7:4:2:2:3)
(D) Isooctane–benzene–acetone–0.15 M triethylammonium sulfate (1:1:1:2)
(E) Methanol–1-propanol–water–toluene–isooctane (4:1:1.4:2:2) (reverse phase)
(F) Isooctane–methanol–water (10:9:1)

Procedure for Incubation Experiments. In an attempt to compensate for the dilution by endogenous mitochondrial cholesterol, more cholesterol sulfate than cholesterol [0.035 μmol of [^{3}H]cholesterol (8.24×10^5 cpm of ^{3}H), and 0.053 μmol of [^{3}H]cholesterol [^{35}S]sulfate (1.48×10^6 cpm of ^{3}H and 3.28×10^6 cpm of ^{35}S; $^3H:^{35}S = 4.5$)] was employed as substrate in expt I. [Simpson *et al.* (1972) reported that the amount of cholesterol in rat adrenal ranges from 5 to 33 nmol per mg of protein, with an average of 16 nmol.] However, since the fraction of the total cholesterol that is available for steroidogenesis is unknown, the dilution of the tracer was ignored in expt II. In this experiment, equal amounts (0.053 μmol) of [^{3}H]cholesterol (8.24×10^5 cpm of ^{3}H) and [^{3}H]-cholesterol [^{35}S]sulfate (1.48×10^6 cpm of ^{3}H and 3.02×10^5 cpm of ^{35}S; $^3H:^{35}S = 4.9$) were used. Two milliliters of solution B was added to each mixture of substrates which previously had been dissolved in 0.1 ml of propylene glycol. The mixtures were shaken for 0.5 hr to ensure complete dispersion. To each solution, 0.5 ml of solution B, containing one of the factors listed in Table III, was added. The reaction was started by the addition of 0.5 ml of the mitochondrial suspension. (Albumin was used to preserve the intact mitochondria; its effect on the enzymatic oxidation of the substrates was not tested.) The final concentrations of the following substances in the reaction mixtures were: *d,l*-isocitrate, 20 mM; succinate, 10 mM; TPNH, 1 mM; and $CaCl_2$, 10 mM (all titrated to pH 7.4 with 1 N NaOH). In expt I, the amount of protein added was 1.74 mg; in expt II, it was 0.96 mg. Incubation was carried out at 37° for either 30 or 60 min. The reaction was stopped by the addition of 6 ml of methanol containing the appropriate carrier steroid. When cholesterol sulfate was the

TABLE II: Crystallization Data of Isolated [^{3}H]Pregnenolone [^{35}S]Sulfate, [^{3}H]Pregnenolone, and [^{3}H]Progesterone.[a]

	Sample	Wt (mg)	^{3}H cpm	^{35}S cpm	^{3}H cpm/mg	^{3}H cpm/^{35}S cpm
[^{3}H]Pregnenolone [^{35}S]sulfate	X-1	1.898	334	78	176	4.3
	ML-1	0.626	257	42	410	6.1
	X-2	1.181	208	50	177	4.2
	ML-2	1.007	190	43	189	4.4
	X-3	1.502	269	61	179	4.4
	ML-3	0.827	166	37	201	4.4
[^{3}H]Pregnenolone	X-1	1.003	34		34	
	ML-1	1.188	86		72	
	X-2	1.988	60		30	
	ML-2	1.056	44		42	
	X-3	3.073	89		29	
	ML-3	0.904	28		31	
[^{3}H]Progesterone	X-1	1.152	37		32	
	ML-1	1.014	187		184	
	X-2	1.028	29		29	
	ML-2	1.294	42		32	
	X-3	1.064	30		28	
	ML-3	0.649	19		29	

[a] The radioactive steroids were products isolated in expt I from the incubation containing no added cofactors. Pregnenolone sulfate was crystallized as its triethylammonium salt: X-*n* = product of the *n*th crystallization; ML-*n* = residue in the mother liquor of the *n*th crystallization.

substrate, 3 mg of the triethylammonium salt of pregnenolone sulfate was added. When cholesterol was the substrate, 3 mg each of pregnenolone and progesterone was used. The aqueous methanolic solutions were filtered, and their precipitates were washed thoroughly with methanol. In those experiments where cholesterol was a substrate, petroleum ether was used as a final wash.

Isolation of Pregnenolone Sulfate from the Incubations with Cholesterol Sulfate. The appropriate filtrate was evaporated under vacuum and chromatographed on Celite in system C, in which pregnenolone sulfate is eluted in the fourth hbv. Those fractions containing pregnenolone sulfate were pooled, and evaporated under vacuum. The residue was converted into the triethylammonium salt (Mickan *et al.*, 1969) and rechromatographed on Celite in system D. The tubes containing pregnenolone sulfate (hbv 3–4) were again combined and evaporated, and an aliquot was taken for quantification by the method of Crépy and Rulleau-Meslin (1960). Negligible amounts of more polar products were found in the methanol wash of the column. An additional quantity of the carrier, the Et_3N salt of pregnenolone sulfate, was added to bring the weight of the sample to 25 mg. The sample was recrystallized first from methanol–ether, then from methylene chloride–ether, then from methanol–ether, and finally from methylene chloride–ether. The specific activities of each crystalline product and of the residue in its mother liquor, with respect to ^{3}H and ^{35}S, and the ^{3}H:^{35}S ratio were determined. The radioactivities of the products and the mother liquor residues (with respect to both ^{3}H and ^{35}S) were such as to indicate that the isotopic moieties were identical with the added cold carrier. The ^{3}H:^{35}S ratios of the products were equal to that of the substrate, cholesterol sulfate, indicating that the conversion had occurred with retention of the sulfate group. The data from a representative crystallization are shown in Table II.

Isolation of Pregnenolone and Progesterone from the Incubations with Cholesterol. The residue obtained from the evaporation of the alcoholic extract of the incubation solution was chromatographed on Celite in the reverse-phase system E. The first hbv contained the more polar steroids such as progesterone, pregnenolone, *etc.*, whereas cholesterol was found in the fourth hbv. After evaporation of the solvents, the residue in hbv 1 was rechromatographed on Celite in system F. The fractions containing progesterone (fourth hbv) were combined, evaporated, and the steroid was quantified by its absorption at 240 nm. Pregnenolone, which was found in the sixth hbv, was quantitatively assayed by the method of Oertel and Eik-Nes (1959). The Celite column was washed with methanol and counted to determine whether more polar compounds were formed. The amount of radioactivity in all these eluates compared to the unincubated control was negligible and was not further investigated. Carrier pregnenolone and progesterone were added so that each sample contained 25 mg, and then each sample was recrystallized at least three times from methanol. The specific activities with respect to ^{3}H of each crystalline product, and of the residues in the mother liquors from each, were determined, and found to be equal to each other. Representative crystallization data are shown in Table II.

The per cent yields of the three steroidal products (pregnenolone sulfate, pregnenolone, and progesterone) were calculated by using the following formula: % yield = $a \times 25 \times (b/c) \times (1/d) \times 100$, where a = specific activity of the product of the final crystallization in cpm/mg; b = weight of carrier added when the reaction was stopped; c = weight of carrier recovered before additional carrier was added to bring the weight of the sample to 25 mg, and d = cpm of ^{3}H or ^{35}S associated with the labeled substrate incubated with the mitochondrial preparation. The results are shown in Table III.

Incubation of Cholesterol and Cholesterol Sulfate with an Acetone Powder Prepared from Rat Adrenal Mitochondria.

TABLE III: Results of Incubations of [1,2-^{3}H]Cholesterol and [1,2-^{3}H]Cholesterol [^{35}S]Sulfate with Mitochondria Isolated from Rat Adrenal Glands.[a]

Additions	Cholesterol Sulfate		Cholesterol					
	PS[b] (pmol/mg of Protein)	PS % Yield	pmol/mg of Protein		P_Δ / ($P_0 + P_\Delta$)	$P_0 + P_\Delta$ (pmol/mg of Protein)	$P_0 + P_\Delta$ % Yield	PS[e] / ($P_0 + P_\Delta$)
			P_Δ[c]	P_0[d]				
Experiment I								
Zero time[f]	5	0.02	2.4	0		2.4	0.01	
None	168 (4.4[g])	0.55	22	31	0.42	53	0.26	2.1
Succinate	288 (4.4)	0.95	41	8	0.84	49	0.25	3.8
Isocitrate	441 (4.4)	1.45						
TPNH	226 (4.5)	0.74						
TPNH + Ca^{2+}	1480 (4.5)	4.9						
Experiment II								
Zero time			4	0		4	0.01	
None			141	89	0.61	230	0.42	
Succinate			194	27	0.88	221	0.40	
Isocitrate	770 (4.7)	1.39	333	38	0.90	371	0.67	2.07
TPNH + Ca^{2+}	1086 (5.0)	1.97	508	50	0.91	558	1.01	1.95

[a] Incubations were performed as described in the text, for 0.5 hr. [b] PS = pregnenolone sulfate. [c] P_Δ = pregnenolone. [d] P_0 = progesterone. [e] Value calculated from per cent yields. [f] Zero time control values were obtained from a mixture that was not incubated. [g] ^{3}H:^{35}S ratio of isolated [^{3}H]pregnenolone [^{35}S]sulfate; ^{3}H:^{35}S ratio of [^{3}H]cholesterol [^{35}S]sulfate was 4.5 in expt I and 4.9 in expt II.

Rat adrenal mitochondria were isolated in a manner similar to that described above except that bovine serum albumin was eliminated from all solutions. The mitochondrial pellet was resuspended in approximately 0.5 ml of water, and the suspension was added to at least 20 volumes of acetone at −20°. The precipitate was collected either by filtration or by centrifugation, washed once with acetone, and then with ether. All procedures were carried out at −20°.

The procedures used for incubation and measurement of side-chain cleavage were those previously described (Hochberg *et al.*, 1974). [26-^{14}C]Cholesterol or [26-^{14}C]cholesterol sulfate was dispersed by dissolving each in acetone containing Tween 80 (Sigma). The acetone was evaporated under N_2 and 0.1 M Tris-HCl buffer (pH 7.4) was added. The concentration of the detergent in this solution was 100 μg/ml. The mixture was sonicated three times for 20 sec at 50 W (sonifier, Model W185D, from Heat Systems, Ultrasonics, Inc., Plainview, N. Y.) using the microtip to disperse the hydrophobic substances. Aliquots of the solutions were taken, and were counted to determine the extent of dispersion. Usually, this was greater than 90%. The two solutions were diluted with the Tris buffer–Tween 80 mixture so that the concentrations of both substrates were equal.

The acetone powder was suspended in 0.1 M Tris-HCl and sonicated four times for 30 sec at 0°. The suspension was centrifuged at 10,000*g* for 10 min, and the supernatant thus obtained was used for incubation. The TPNH-generating system consisted of glucose-6-PO_4 (2.5 mg), glucose-6-PO_4 dehydrogenase (0.5 U), and $MgCl_2$ (95 μg), dissolved in 0.1 ml of 0.1 M phosphate buffer (pH 7.4). The incubation was performed by preincubating 0.1 ml of the solution containing the sterol substrate for 2 min at 30° with 0.8 ml of the acetone powder supernatant (300 μg of protein). The reaction was started by adding 0.1 ml of the TPNH-generating system, which has also been preincubated at 30° for 5 min. The final concentration of Tween 80 in all incubations was 10 μg/ml. The incubation was carried out at 30°. Aliquots (0.2 ml) were taken at zero time and every 2 min thereafter for 8 min. Each aliquot was diluted with 1.5 ml of 0.001 M $HgCl_2$ in 0.05 M glycine–NaOH buffer (pH 9.5). This mixture was percolated through a micro alumina column, and the amount of C_6 fragment formed was determined from the radioactivity in the eluate. The velocity was calculated (by computer) by linear regression of the experimentally determined values. In this manner the apparent K_m of the two substrates was measured by varying the concentration of each (Figure 1).

Inhibition Studies. The effect of cholesterol sulfate on the oxidation of [26-^{14}C]cholesterol and the effect of cholesterol on the cleavage of [26-^{14}C]cholesterol sulfate were studied, and the results are depicted in Figure 2. Acetone powders of bovine adrenal mitochondria were prepared essentially in the same manner as those derived from rat mitochondria with one modification. Both the medulla and the capsule were discarded before the tissue was homogenized. The radioactive substrate (2.7 nmol) was incubated with 400 μg of the bovine adrenal mitochondria preparation in the presence of varying

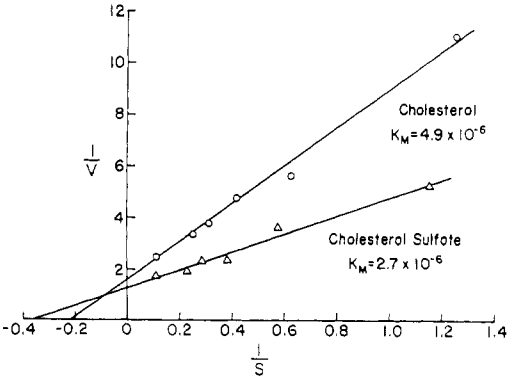

FIGURE 1: Lineweaver–Burk plot of the side-chain cleavage of cholesterol and cholesterol sulfate. V = nmole × 10^{-1} min, S = 10^{-6} M. Acetone powder from rat adrenal mitochondria was the source of the enzyme.

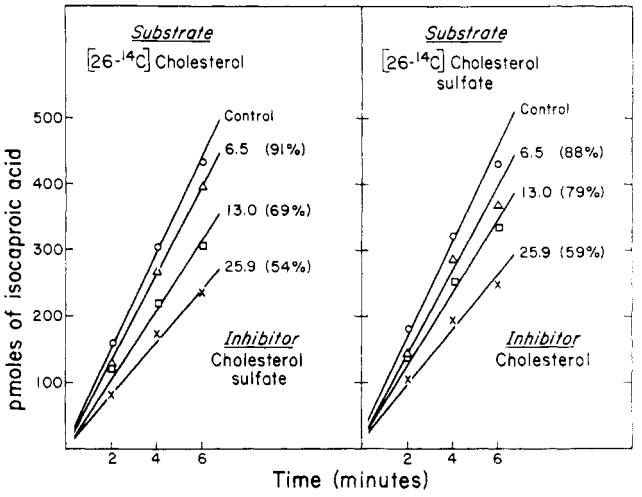

FIGURE 2: Inhibition of the side-chain cleavage of [26-^{14}C]cholesterol by cholesterol sulfate and of [26-^{14}C]cholesterol sulfate by cholesterol. Acetone powder from bovine adrenal mitochondria was the source of the enzyme. The concentration of both substrates was 2.7×10^{-6} M. The values at the end of each line are the concentrations of inhibitor, in μM. The values in brackets are the rates of reaction expressed as a per cent of the control.

amounts of its unlabeled relative. Both the free and the conjugated sterols were solubilized in Tween 80 whose concentration in these experiments was 20 μg/ml. The rate of side-chain cleavage was determined every 2 min for 6 min by measuring the C_6 fragment formed as described above.

Sodium cholesterol glucuronide was also tested as an inhibitor of the side-chain cleavage enzyme with free cholesterol and its sulfate as substrates, and the results of this experiment are shown in Table IV.

Effect of Succinate upon Sterol Side-Chain Cleavage. The side-chain cleavage assay (Hochberg *et al.*, 1974) was used to study further the effects of succinate upon the oxidation of cholesterol and of cholesterol sulfate by intact mitochondria. Samples were taken every 15 min for 1 hr, and the amount of the ^{14}C side-chain fragment liberated was determined. The rate of reaction was linear in all cases during this interval. The rates of oxidation of the two substrates were measured both in the presence and in the absence of succinate, and the results are described below.

Results

The results of the incubation of the sterols with intact mitochondria (expt I and II) are presented in Table III. The rate of reaction was linear for at least 1 hr. Except for the zero-time values, all the data listed in Table III were obtained with incubations carried out for 0.5 hr. Also shown in this table are the ^{3}H:^{35}S ratios of the final recrystallizations of pregnenolone sulfate. As is evident, the ratios are virtually identical with that of the substrate, [^{3}H]cholesterol [^{35}S]sulfate, proving that the sulfate moiety is retained throughout the conversion. The data in Table III also demonstrate that even in the absence of additives, there are sufficient endogenous reducing equivalents in the mitochondria, either in the form of the endogenous TPNH or its energy-linked substrates, to support steroidogenesis, albeit at a rate lower than that occurring when some cofactors were added.

The data in Table III also show that the addition of TPNH alone to mitochondria only slightly increases the conversion of cholesterol sulfate into pregnenolone sulfate. However, when mitochondria are treated with calcium ions (Hirshfield and Koritz, 1964), the exogenously added nucleotide alone was then able to support side-chain cleavage presumably because the membrane becomes permeable to TPNH. The small increase found when TPNH alone was added (35%) may indicate that some of the mitochondria have been damaged. However, the much greater increase (9-fold) in side-chain cleavage caused by TPNH when the mitochondrial membrane is disrupted by Ca^{2+} is evidence that the vast majority of mitochondria were intact under the usual conditions of this experiment. Of all the additives listed in Table III, TPNH, under the conditions defined, stimulated the reaction most.

TABLE IV: Effect of Cholesterol Glucuronide on Rates of Side-Chain Cleavage of [26-^{14}C]Cholesterol Sulfate and [26-^{14}C]Cholesterol.[a]

Cholesterol Glucuronide (μM)	Cholesterol		Cholesterol Sulfate	
	Velocity (pmol/min)	% of Control	Velocity (pmol/min)	% of Control
0	73.5		65.0	
6.5	72.8	99	59.1	91
12.9	67.2	91	59.6	92
25.8	73.8	100	66.7	103

[a] Acetone powder from bovine adrenal mitochondria was the source of the enzyme. The concentration of each substrate was 2.7×10^{-6} M.

Isocitrate increased the side-chain cleavage of both the free and sulfated sterols although less than TPNH and Ca^{2+}. On the other hand, isocitrate accelerated the rates of oxidation of free and sulfated cholesterol more than when TPNH alone or succinate alone were added to the incubation mixture.

The addition of succinate to the incubation mixture containing adrenal mitochondria did not increase the rate of formation of C_{21} steroids from cholesterol: per cent yield of progesterone + pregnenolone, 0.26 without succinate and 0.25 with succinate in expt I and 0.42 without succinate and 0.40 with succinate in expt II. On the other hand, when cholesterol sulfate was the substrate, the addition of succinate increased the per cent yield of pregnenolone sulfate from 0.55 to 0.95 (expt I). The addition of succinate affected the ratio of the products from cholesterol (progesterone and pregnenolone) although the total amount of C_{21} steroids formed was not altered. In both expt I and II, the presence of succinate increased the amount of pregnenolone that was isolated over that obtained in its absence. Contrariwise, the amount of progesterone isolated in the presence of succinate was less than that found in its absence. When succinate was added, the total amount of C_{21} steroid synthesis was maintained, but the oxidation of pregnenolone to progesterone was markedly inhibited. The inhibition of this oxidation is probably due to the indirect reduction by succinate of DPN, an essential cofactor for the 3β-hydroxy-Δ^5-dehydrogenase system.

To extend these observations, the rates of side-chain cleavage of cholesterol and cholesterol sulfate were restudied by measuring the amount of [^{14}C]isocaproic acid formed from [26-^{14}C]sterols, both in the presence and in the absence of succinate, using adrenal mitochondria as the source of cleavage enzyme. When cholesterol was the substrate, the ratio of the velocity of the oxidation in the presence of succinate to that observed in its absence, was 0.76. When cholesterol sulfate was the substrate, the ratio was 1.83. Duplicate determinations obtained in these experiments were 0.77 and 1.77,

respectively. The effect of succinate on oxidation was again determined using a different mitochondrial preparation, and the ratios observed (in duplicate) were 0.84 and 0.84 for cholesterol and 1.69 and 1.65 for cholesterol sulfate. Thus, the addition of succinate to these preparations appeared to cause a decrease in the rate of oxidation of [26-^{14}C]cholesterol and an increase in the rate of oxidation of the 26-^{14}C-labeled sterol sulfate.

Table III also reveals that, regardless of the conditions used in the incubations, the percentage yield of radioactive pregnenolone sulfate from cholesterol sulfate was considerably greater than was the yield of C_{21} steroids (pregnenolone + progesterone) from cholesterol. In spite of the fact that the ratio of the concentrations of cholesterol sulfate and cholesterol in expt I was different from that used in expt II, the ratios of the products, pregnenolone sulfate:pregnenolone + progesterone (last column Table III), when calculated on a percentage yield basis, were equal.

Consistent with these observations are the kinetic data obtained with acetone powders as depicted in Figure 1; at all concentrations tested the rate of oxidation of cholesterol sulfate was faster than that of cholesterol. The rates of side-chain cleavage of the two substrates were measured, as described above, by determining the amount of [^{14}C]isocaproic acid formed. The reaction rates were linear during the 8-min incubation period.

While the data in Figure 1 are representative, they are not characteristic of all the kinetic experiments carried out during this study. With enzyme preparations made from rat adrenals, the results were reasonably consistent but this was not true when bovine adrenals were the source of the enzyme. Thirty-six experiments in which rates were determined were performed using 35 different acetone powders prepared from rat adrenal glands. In the majority of these, 33 out of 36, the rate of cleavage of cholesterol sulfate was greater than that of cholesterol. With one preparation, the rates were equal, and, with two, the rate of oxidation of cholesterol sulfate was less than that of cholesterol. On the other hand, when acetone powders made from bovine adrenals were used, the variability was greater. In 18 experiments, using 5 different powders, cholesterol sulfate was oxidized at a rate faster than was cholesterol in 9. In two, the rates were equal, and, in 7, the sulfate was cleaved at a slower rate than was the free sterol. As of now, this variability is unexplained. Whatever the cause of the inconsistencies, the fact remains that the rate of cleavage of cholesterol sulfate is at least equal to, and most probably faster, than that of the free sterol.

The apparent K_m of cholesterol sulfate, calculated from the data shown in Figure 1, is less than that of cholesterol. Although not conclusive, these data suggest that the sulfate is bound more tightly to the enzyme than is the free sterol.

Inhibition studies proved that either substrate could diminish the rate of cleavage of the other. When [26-^{14}C]-cholesterol (2.7 nmol) was incubated with an acetone powder of bovine adrenal mitochondria, a TPNH-generating system, and varying amounts of cholesterol sulfate, the rate of oxidation of the sterol was reduced (Figure 2). Analogously, when the substrate was [26-^{14}C]cholesterol sulfate and the inhibitor, cholesterol, the rate of oxidation of the sulfate was suppressed (Figure 2). No desulfation of cholesterol sulfate occurred under the conditions of these experiments, and, therefore, the inhibition of cleavage cannot be accounted for by dilution of the radioactive substrates by sterol formed in this manner. When cholesterol glucuronide was added to the incubation mixtures in the concentrations given in Table IV, no alteration in the rate of oxidation of either cholesterol or its sulfate was observed.

Discussion

That cholesterol sulfate like cholesterol itself is capable of penetrating the membrane of the adrenal mitochondria has special relevance for the importance of this conjugate as a biosynthetic precursor of steroid hormones. If it had been true, as was suggested by Roberts *et al.* (1967), that the mitochondrial membrane is impermeable to the sterol sulfate, then it might appear that cholesterol sulfate is not of any great significance as a substrate for hormone synthesis even though it has been possible to demonstrate its conversion into pregnenolone sulfate by a mitochondrial enzyme. The cleavage enzyme is found in the inner membrane of this subcellular particle (Yago and Ichii, 1969) and since the conjugate is not synthesized within the mitochondria (Roy, 1970) its impermeability would tend to suggest that it was not a quantitatively important precursor. But the data given in Table III show that cholesterol sulfate is converted into pregnenolone sulfate by intact mitochondria. The failure of Roberts *et al.* (1967) to observe this conversion was most probably due to their use of TPNH as a source of reducing equivalents. Mitochondria are known to be impermeable to TPNH and DPNH (Lehninger, 1964). Indeed, this criterion was used in the present experiments to demonstrate that the mitochondria were intact. The data in Table III show that exogenously added TPNH does not substantially increase the rate of oxidation of the sterol sulfate unless the mitochondria are disrupted with some agent such as Ca^{2+}.

The first proof that cholesterol sulfate can be converted into various steroid sulfates was obtained *in vivo* when this sterol conjugate was infused during surgery directly into a vein leading to an adrenal tumor (Roberts *et al.*, 1964). It was assumed that the cleavage reaction had occurred because the mitochondria in the diseased tissue were "leaky." This contrived speculation is now made unnecessary by the demonstration in this report that the conjugate can, in fact, penetrate intact mitochondria. Thus, cholesterol sulfate originating in other regions of the adrenal gland or perhaps even available from circulating blood can serve as a hormonal precursor.

It has been postulated (Young and Hall, 1969; Young *et al.*, 1970) that there is one enzyme that cleaves the side chain of cholesterol and another which oxidizes its sulfate. This issue is obviously important and, if true, could have far reaching implications. Young and Hall (1969) as well as Kobayashi and Ichii (1969) have described inhibition experiments which appear to lend support to the existence of two enzymes. The observations reported in the present paper are at variance with their findings for our results show that cholesterol sulfate inhibited the enzymatic oxidation of cholesterol and cholesterol inhibited the oxidation of cholesterol sulfate. The reason for the discrepancy is not evident. While our findings are in accord with those of Raggatt and Whitehouse (1966), neither distinguishes between these two possibilities, one enzyme that catalyzes the oxidation of both substrates or one enzyme specific for each of the two substrates. Analysis of the data accumulated at this time leads us to the conclusion that this question is still unresolved. The results obtained by Jacobsohn and Hochberg (1968) may be relevant for they found that the 17β-hydroxysteroid dehydrogenase present in human red blood cells catalyzes the oxidation of both estradiol and its sulfate.

The fact that the addition of succinate affected differently the rates of oxidation of cholesterol and cholesterol sulfate

might be interpreted as support for the existence of two separate enzymes. The addition of this dicarboxylic acid accelerated the rate of side-chain cleavage of cholesterol sulfate (using mitochondria from rat adrenal glands) whereas it altered the rate of oxidation of the free sterol only slightly. However, other explanations of these results are possible, *e.g.*, succinate stimulates the transport of cholesterol sulfate across the mitochondrial membrane without effecting the diffusibility of cholesterol or succinate stimulates the release of cholesterol from the mitochondrial membrane thus diluting the radioactive substrate. This latter event would exhibit itself as an apparent deceleration of the rate of oxidation of the labeled sterol precursor.

The results shown in Table III suggest that cholesterol sulfate is converted into pregnenolone sulfate at a rate at least comparable to, and probably greater than, that by which cholesterol is converted into its C_{21} products. Because of the unknown dilution by unlabeled endogenous substrates, and the uncertainty caused by the high concentrations of exogenous substrates employed, the results of these kinetic experiments may be illusionary. Consequently, the rates of oxidation of cholesterol and cholesterol sulfate were estimated at a variety of substrate concentrations when adrenal mitochondrial acetone powders were used as the source of the enzyme. Because acetone powders presumably contain neither cholesterol nor cholesterol sulfate, the estimated initial rates should be independent of uncertain quantities of endogenous substrates. However, cholesterol does remain in the mitochondrial powder as further extraction of the acetone powder with boiling solvents demonstrated. Acetone powders prepared from adrenals were found by gas–liquid chromatography to contain somewhat less than 0.5 nmol of cholesterol/mg of protein. Consequently, in a usual incubation which contained 200–400 μg of protein, the amount of endogenous cholesterol present in the acetone powder was less than 10% of the added ^{14}C-labeled substrate and would not be expected to alter the results appreciably. As pointed out above, the finding that cholesterol sulfate is oxidized at a faster rate than is cholesterol was more often observed when enzyme preparations made from rat adrenals were used than when bovine adrenals served as the source of the enzyme. The factors that determined these irregularities are unknown.

Acetone powders were also used to determine the apparent K_m values of cholesterol and cholesterol sulfate. The data plotted in Figure 1 reveal that the apparent K_m of cholesterol sulfate is smaller than that of cholesterol, and also that the V_{max} for cholesterol sulfate is greater than that of the free sterol. It is clear, therefore, that these results using acetone powders of adrenal mitochondria confirm those of expt I and II in which intact mitochondria were the enzyme source. Raggatt and Whitehouse (1966) also measured the apparent K_m's for cholesterol and cholesterol sulfate, and reported that the value obtained for cholesterol was less than that for its sulfate. However, these investigators used bovine mitochondria as their enzyme source and since this cellular subfraction unquestionably contains endogenous sterol, K_m values calculated from experiments using intact mitochondria must be of uncertain value. Using a partially purified cleavage enzyme isolated from bovine adrenals, Young and Hall (1969) also measured K_m values and, like our own, their results indicated that cholesterol sulfate has a smaller K_m than free cholesterol.

Although the results reported in this paper indicate that cholesterol sulfate is oxidized at a rate comparable to or faster than that of cholesterol, and that the conjugate appears to be bound to the enzyme somewhat better than is the free sterol, it remains difficult to attribute to these findings significance that holds for *in vivo* conditions. The hydrophobic nature of the substrates, artificially dispersed by sonication and detergents, is obviously an important unknown factor in determining the validity of these kinetic measurements. In addition, the amounts of C_{21} products, pregnenolone or pregnenolone sulfate formed under natural circumstances, obviously depend upon the endogenous pool sizes of the respective precursors, and these are difficult to estimate. It is likely that both cholesterol and its sulfate serve many functions in the adrenal and determination of the sizes of the only relevant pools, those which are "active" for steroidogenesis, is, at present, impossible. Another important point that must be considered is that the kinetic parameters, the initial rates, and apparent K_m values, were determined for both substrates using acetone powders of adrenal mitochondria. To derive meaningful conclusions simply by comparing these kinetic parameters would require the assumption that the cleavage enzyme preparation is homogeneous, and is derived from one source. Of course, the preparation is derived from adrenal glands, but these are well known to contain several different types of steroidogenic cells. The adrenals synthesize, from C_{27} sterols, and secrete, C_{21} glucocorticoids, C_{21} mineralocorticoids, C_{19} steroids (such as dehydroisoandrosterone sulfate, testosterone, and androstenedione), and even C_{18} estrogens. The synthesis of each of these classes of steroids has been traditionally associated with a different cell type, or zone of the adrenals, and, even if the evidence for this association is far from certain, it is logical to believe that each class of product is synthesized within its own specialized cell. It would seem unreasonable to suppose that each cell can biosynthesize all classes of steroidal products. If this analysis is correct, there is no reason to believe that the mitochondria of all the steroid-producing cells are alike, and, particularly, alike in their substrate requirements. Obviously, the adrenal mitochondria used by us in this study, and by all other workers in their investigations, must be considered to be inhomogeneous, and it is not far fetched to suppose that some mitochondria use cholesterol as a precursor for their specific products and others use cholesterol sulfate as their substrate.

Moreover, if it is true that adrenal mitochondria from rats oxidize cholesterol and cholesterol sulfate differently than do adrenal mitochondria from beef, as our preliminary data imply, then species is another factor that must be taken into consideration in evaluating the significance of the results reported herein. This is especially true for humans where it is known (Roberts and Lieberman, 1970) that the adrenal is rich in cholesterol sulfate. In this connection, it is especially noteworthy that one of the quantitatively most important secretory products of the human adrenal is dehydroisoandrosterone sulfate, a conjugate known to be formed from cholesterol sulfate.

Because of these considerations, it is not possible to extrapolate accurately the results obtained in this study to *in vivo* circumstances. Nevertheless, the findings reported here suggest that cholesterol sulfate, unlike other known naturally occurring esters of cholesterol, deserves serious attention as an important precursor of at least some of the steroid hormones.

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Comparison of Proteins of Ribosomal Subunits and Nucleolar Preribosomal Particles from Novikoff Hepatoma Ascites Cells by Two-Dimensional Polyacrylamide Gel Electrophoresis†

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ABSTRACT: Proteins have been isolated from Novikoff hepatoma polyribosomes, ribosomal subunits, and nucleolar preribosomal particles and compared by two-dimensional polyacrylamide gel electrophoresis. The large and small ribosomal subunits contain 35 and 23 proteins, respectively. Nucleolar particles contain approximately 60 proteins, 21 of which are present in the large ribosomal subunit and 10 of which are present in the small ribosomal subunit. Nucleolar preribosomal particles isolated in the presence of poly(vinyl sulfate) contain specific proteins which are removed when these particles are isolated in media containing sodium ethylenediaminetetraacetate. One of these proteins, B 13′, has identical electrophoretic mobilities in two-dimensional gel electrophoresis to a protein removed from ribosomes during dissociation of ribosomes into subunits with sodium ethylenediaminetetraacetate. A processing mechanism is proposed for the removal of nucleolar preribosomal associated proteins not present in cytoplasmic ribosomes by which some of these proteins are conserved in the nucleolus.

It has been well established that the nucleolus is the site of ribosomal precursor RNA synthesis (Perry, 1962; Scherrer and Darnell, 1963; Darnell, 1968; Busch and Smetana, 1970). Several studies (Tamaoki, 1966; Warner and Soeiro, 1967; Liau and Perry, 1969; Mirault and Scherrer, 1971; Auger and Tiollais, 1973) have shown that newly transcribed 45S ribosomal precursor RNA becomes associated with protein and can be isolated as a rapidly sedimenting ribonucleoprotein complex. It has also been shown that the granular component of the nucleolus is largely composed of ribonucleoprotein complexes which largely are precursors to the large ribosomal subunit (Shankarnarayan and Birnstiel, 1969; Busch and Smetana, 1970; Das *et al.*, 1970; Koshiba *et al.*, 1971). There are many common proteins in nucleolar ribonucleoprotein particles and the large ribosomal subunit (Kumar and Warner, 1972; Tsurugi *et al.*, 1973).

The nature of the nucleolar precursor to the small ribosomal subunit is not clear although studies of tryptic digestion products of nucleolar proteins (Shepherd and Maden, 1972) suggest that the nucleolar ribonucleoprotein particles containing 45S RNA also contain proteins of the small ribosomal subunit.

Since it has been shown that an increased resolution of proteins of mammalian ribosomes (Kaltschmidt and Wittman, 1970; Martini and Gould, 1971; Welfle *et al.*, 1971; Delaunay and Schapira, 1972; Delaunay *et al.*, 1972; Hulten and Sjoquist, 1972; Sherton and Wool, 1972; Welfle *et al.*, 1972; Rodgers, 1973), nuclei (Yeoman *et al.*, 1973), and nucleoli (Ballal and Busch, 1973; Orrick *et al.*, 1973) can be obtained by two-dimensional polyacrylamide gel electrophoresis, a

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