Cholesterol Sulfate Inhibits Adrenal Mitochondrial Cholesterol Side Chain Cleavage at a Site Distinct from Cytochrome P-450<sub>sec</sub>

EVIDENCE FOR AN INTRAMITOCHONDRIAL CHOLESTEROL TRANSLOCATOR*

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Cholesterol sulfate inhibits (K<sub>50</sub>, 6 μM) the side chain cleavage of exogenous cholesterol in intact rat adrenal mitochondria. Inhibition is at a site other than cytochrome P-450<sub>sec</sub>; the spin state of the hemoprotein is not perturbed, and its activity is unaffected as judged by the failure to inhibit the metabolism both of 25-hydroxycholesterol and of endogenous cholesterol in a mitochondrial “steroidogenic pool.” In contrast, 25-hydroxycholesterol, known to interact with the cytochrome, prevented the cleavage of both endogenous and exogenous cholesterol and produced the expected optical changes in the hemoprotein. Inhibition was specific, since a variety of related compounds including pregnenolone sulfate were not effective. Metabolic conversion to other species was insufficient to account for inhibition, indicating that cholesterol sulfate is the effective molecule. A hallmark of an inhibitor of a transport system is that disruption of the barrier to transport eliminates inhibition. Sonic disruption of mitochondria abated 70% the effect of cholesterol sulfate, but did not affect inhibition by 25-hydroxycholesterol. Thus, the cholesterol sulfate appears to inhibit an intramitochondrial cholesterol translocation system that functions to move cholesterol into a steroidogenic pool. The high content of cholesterol sulfate in adrenal cortex (Drayer, N. M., Roberts, K. D., Bandi, L., and Lieberman, S. (1964) J. Biol. Chem. 239, 3112-3114) suggests a possible regulatory role for this molecule.

The side chain cleavage of cholesterol to yield pregnenolone is the initial and rate-limiting step in steroid hormone biosynthesis in steroidogenic tissues, including the adrenal cortex. The reaction is catalyzed by a mitochondrial enzyme system consisting of cytochrome P-450<sub>sec</sub> (ssc for side chain cleavage), together with its NADPH-specific electron transport system (NADPH-adrenodoxin reductase and adrenodoxin; reviewed in Ref. 1). The cytochrome is an integral membrane protein (2, 3), associated with the inner aspect of the inner mitochondrial membrane (4-5), and appears to utilize as its substrate membrane-dissolved rather than aqueous cholesterol (2, 6).

Side chain cleavage in the adrenal cortex is acutely stimulated (within 5-10 min) by adrenocorticotropic hormone (ACTH), a pituitary peptide hormone released in response to stress. Activation requires protein but not RNA synthesis since cycloheximide (and other inhibitors of protein synthesis), but not inhibitors of RNA synthesis, prevents activation (7, 8). The cycloheximide-inhibitable, ACTH-regulated step is expressed in the mitochondrion (9), since the activation state is preserved in isolated mitochondria from pretreated animals. Ether anesthesia (“ether stress”) can be used as in the present studies to elevate ACTH levels in vitro (10) and allows subsequent isolation of steroidogenically active mitochondria (11).

Recent interest in ACTH regulation has focused on two major areas: 1) the identity and properties of the cycloheximide-inhibitable steroidogenic factor, and 2) the mechanism of activation of steroidogenesis within adrenal mitochondria. Regarding the former, Pedersen and Brownie (12) have recently isolated an ACTH-induced 2.2-kDa peptide from adrenal cytosol (termed the Steroidogenic Activator Peptide), which can activate cholesterol side chain cleavage in mitochondria isolated from cycloheximide-pretreated rats. Although other steroidogenic activating factors have been reported and may play important roles in the metabolism of adrenal cholesterol (these include lipids (13, 14) and sterol carrier proteins 1 and 2 (15, 16)), this peptide now appears to be the most likely candidate for the physiological activator.

Regarding the mechanism of activation, most studies have focused on establishing differences between activated versus nonactivated mitochondria. A variety of approaches have led to the conclusion that activation does not directly involve the cytochrome P-450 enzyme system, but may involve the activation of intramitochondrial steroid movement. Early studies by Simpson and colleagues (11) identified two kinetic “pools” for pregnenolone formation in isolated activated mitochondria; these workers suggested that ACTH regulation may involve cholesterol movement into the “steroidogenic pool.” A “barrier” to cholesterol utilization in nonactivated mitochondria was found to limit cholesterol utilization, and mitochondrial disruption (e.g. sonication, calcium-induced swelling) leads to activation of side chain cleavage, presumably by disrupting this barrier (17, 18). Several groups have studied the correlation between the mitochondrial activation state and substrate binding to cytochrome P-450, as assayed by the quantity of this hemoprotein in the high spin state (9, 19, 20). Activation was correlated with an increase in the high spin content, consistent with an increased cholesterol in the steroidogenic pool. Additional aspects of the ACTH mechanism have been reviewed recently (18, 21).

Our earlier kinetic studies using membrane-reconstituted

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†† The abbreviations used are: ACTH, adrenocorticotropic hormone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.
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cytochrome P-450 mc led to the prediction that the steroidogenic pool is inner membrane-dissolved cholesterol (6), and we have proposed (21, 22) that because of the poor water solubility of cholesterol, the aqueous intermembrane space is the barrier to cholesterol movement and utilization. Direct measurements by Privale et al. (23) of cholesterol levels in separated inner and outer mitochondrial membranes provided evidence that the ACTH-regulated, cycloheximide-inhibitable step involves translocation of cholesterol from the outer to the inner membrane. One working hypothesis is that the Steroidogenic Activator Peptide activates this cholesterol redistribution. However, the peptide does not appear to be a cholesterol transport protein since cholesterol binding has not been observed.

Implicit in this scheme is the existence of an ACTH-activatable intramitochondrial cholesterol translocation system. We reasoned that if such a system exists, it is likely to have a somewhat different specificity for steroids than does cytochrome P-450 mc. We had characterized previously the binding specificity of the purified, milliequivalent cytochrome P-450 mc, using a series of cholesterol derivatives (24, 25). In the present studies, we tested these and other steroids for their ability to inhibit the side chain cleavage of cholesterol in activated adrenal mitochondria from ether-stressed rats. We find that inhibition does not parallel the ability of steroids to bind to purified cytochrome P-450 mc. In particular, cholesterol sulfate is a potent inhibitor of mitochondrial side chain cleavage and acts at a site other than cytochrome P-450 mc. Data support the notion that this site is an intramitochondrial cholesterol translocation system.

EXPERIMENTAL PROCEDURES

Materials

Cholesterol was obtained from Applied Science Laboratories. Antibody specific for pregnenolone was purchased from Radioassay Systems Laboratories. Chlo- rosulfonic acid was from Aldrich. Sodium dodecyl sulfate was from Bio-Rad, and cholesterol propyl ether was a gift from Enrico Forcielli of Syntex Research, and cyanoketone (2-cyano-4,4,17α-trimethyl-17β-hydroxyandrostan-5-en-3-one) was a gift of the Sterling-Winthrop Research Institute.

Methods

Mitochondrial Preparation—Adrenals were dissected from male rats (Sprague-Dawley, approximately 200 g) decapitated following 10 min of ether anesthesia. In some cases, animals were preinjected with 10 mg of cycloheximide, 35 min prior to anesthesia. Adhering fat on adrenals was removed, and adrenal tissue extruded from a slice in the adrenal capsule. Mitochondria were isolated by the method of Toaff et al. (26), modified so that the initial buffer used during homogenization and centrifugation steps consisted of 0.25 M sucrose, 1 mM EDTA, 25 mM Hepes, and 10 mg/ml bovine serum albumin, at pH 7.0. After isolation by differential centrifugation, mitochondria were resuspended in the same buffer without bovine serum albumin, recen trifuged, and finally resuspended in incubation buffer (125 mM KCl, 5 mM MgCl2, 10 mM KP, 25 mM Hepes, 0.2 mM EDTA, pH 7.0). Protein was quantified according to Lowry et al. (27). Mitochondrial integrity was tested by permeability to pyridine nucleotides, as described previously (28). In some experiments, mitochondria were sonicated for 5–10 min in a 13 × 100 test tube, using a Beuhler Ultramett III bath-type sonicator.

Enzyme Preparation—Adrenodoxin, adrenodoxin reductase, and cytochrome P-450 mc were isolated from beef adrenal cortex as described previously (6, 29).

Assay of Radiolabeled Cholesterol Sulfate—[3H]Cholesterol (1 mCi; 1.7 μg) was dried from chloroform and redissolved in 0.5 ml of dry pyridine. Cholorosulfonic acid (50 μl) in 0.5 ml of dry pyridine was added to the cholesterol solution with continuous stirring and heated at 80 °C for 15 min. Extracts and further workup are as described in Ref. 30 except that the cholesterol sulfate was redissolved in a final volume of dichloromethane (65:35) and then purified by reversed-phase (C-18) HPLC, using the same solvent containing 0.025% octylamine as an ion pairing reagent.

Mitochondrial Incubations—Incubations were carried out in incubation buffer containing 5 μM cyanoketone and 0.65 mM NADP+, and 4 mM malate was added to initiate the reaction (28). When the appearance of radiolabeled pregnenolone was to be monitored, mitochondria were “preloaded” with radiolabeled cholesterol (0.3 μCi) by incubation at 37 °C for 30 min, at which time cholesterol uptake is maximal, followed by resolation of mitochondria to remove unincorporated cholesterol (31). In other experiments, cholesterol was added immediately prior to initiation of the reaction. In both protocols, concentrated cholesterol was added from an ethanol stock to give a final ethanol concentration of less than 0.6%; in control experiments, this concentration of solvent did not affect side chain cleavage. After incubation (100–200 μl) at time points designated, mitochondria were transferred into vials containing 1 ml of extraction solvent (dichromomethane for radiometric and gas chromatographic assays or hexane for the radioimmunoassay), and mixed by vortexing for 30 s. In some experiments, no cholesterol was added to mitochondria, and pregnenolone production from endogenous cholesterol was monitored (see below).

For incubations with sonicated mitochondria, excess adrenodoxin (1.3 μM) and adrenodoxin reductase (0.5 μM) were added, since dilution of the endogenous enzymes into the incubation volume otherwise makes this electron transport system rate-limiting (28). In addition, an NADPH-generating system was included and consisted of NADPH (120 μM), glucose 6-phosphate (0.5 mM), and glucose-6-phosphate dehydrogenase (0.5 units/ml), in addition to cyanoketone as above.

Asays for Pregnenolone and Cholesterol—Three methods were used for the assay of cholesterol side chain cleavage; each was appropriate to specific experimental circumstances, as described below. The conversion of radiolabeled cholesterol to pregnenolone was quantified by the HPLC method of Tribble et al. (31). Briefly, an aliquot of dichloromethane extract was dried, redissolved in acetonitrile and chromatographed on a reversed-phase C-18 column pre-equilibrated with the same solvent system. Pregnenolone production was monitored by simultaneous radiometric quantitation, using a FLO-ONE Model HP radioisotope flow detector which received eluant directly from the column. Pregnenolone production was expressed as a percent of the total counts in the pregnenolone (4.3 min) and cholesterol (7.3 min) peaks (1.2 ml/min flow rate).

When it was necessary to quantify the mass of pregnenolone rather than the percent conversion, either a radioimmunoassay or gas chromatography was used. Gas chromatography was carried out at 290 °C using a Perkin-Elmer Model 8310 gas chromatograph equipped with a 6-foot glass column (2 mm inner diameter, 3% SP-2250 on 100/120 mesh Supelcoport). Stigmastanol (2.4 mmol) was included in the dichloromethane extraction solvent as a recovery and internal standard. An aliquot of dichloromethane extract was dried, redissolved in acetonitrile/hexane (98:2) and analyzed by gas chromatography; areas were corrected for a 5% FWHM of a nominal 7.4 μM pregnenolone peak. In this experimental setting, a radioimmunoassay for pregnenolone was used, as described previously (32, 33).

Assay of Side Chain Cleavage of Cholesterol Sulfate—Cholesterol sulfate cleavage activity of adrenal mitochondria was monitored as described (21). In mitochondria (2 mg/ml) were predoped with [3H] cholesterol (2 μg/ml) and bladder cutaneous (29) as above with cholesterol sulfate (22 μM) containing 1.2 × 106 cpm of the labeled compound. The reaction was initiated with malate as described above, and the incubation contained 4 μM cyanoketone. Aliquots were taken at various times up to 60 min, pipetted
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RESULTS

Inhibition by Cholesterol Sulfate of Pregnenolone Production from Exogenous [3H]Cholesterol—We initially screened 16 cholesterol analogues and related compounds (see Table I) for their ability to inhibit at 40–50 μM the side chain cleavage of tritiated cholesterol which had been pre-incorporated (see "Experimental Procedures") into adrenal mitochondria. Fig. 1 shows a time course for pregnenolone formation and demonstrates that cholesterol sulfate at 44 μM completely inhibits the formation of radiolabeled pregnenolone. The concentration for half-maximal inhibition using this assay was 6 μM (see below). Of the other steroids tested (which included other cholesterol esters), only 25-hydroxycholesterol showed significant (80%) inhibition at similar concentrations (see Table I). Inhibition by 25-hydroxycholesterol was not unexpected, since it is known to be a good substrate for the side chain cleavage enzyme (34, 35) and should compete at the steroid binding site (24, 25) for metabolism of the tritiated cholesterol.

Specificity of Inhibition by Cholesterol Sulfate—To investigate the specificity of inhibition by cholesterol sulfate, other sulfated hydrophobic compounds were tested (see Table I). At a concentration similar to that of cholesterol sulfate which completely (greater than 99%) inhibited side chain cleavage, sodium dodecyl sulfate inhibited only 35%. Pregnenolone sulfate at a similar concentration produced 26% inhibition, somewhat less than that seen with sodium dodecyl sulfate. The specificity was even more apparent at 22–24 μM, where cholesterol sulfate inhibited greater than 90% of activity, but pregnenolone sulfate was completely ineffective. Because pregnenolone sulfate has the same ring structure and stereochemistry as cholesterol, and differs only in the replacement of the cholesterol side chain with an oxygen, the inhibition appears to show a great deal of specificity for cholesterol sulfate.

Effects of Cholesterol Sulfate on the Rapid and Slow Phases of Pregnenolone Formation—Simpson et al. (11) and others (28, 36, 37) have shown previously that when an exogenous source of cholesterol is provided to adrenal mitochondria and the total pregnenolone production is quantified, there is an initial rapid phase of pregnenolone production which occurs within the first few minutes, followed by a slower phase lasting at least 30 min. These data have generally been interpreted as reflecting the initial cleavage of cholesterol in a steroidogenic pool (i.e. one which is accessible to the side chain cleavage enzyme), followed by a slower movement of cholesterol into this steroidogenic pool.

Fig. 2 shows that when the total pregnenolone production (i.e. that from both endogenous and exogenous sources of cholesterol) is quantified using a radioimmunoassay, the slow phase is inhibited by cholesterol sulfate, whereas the initial burst was relatively unaffected. The size of the burst phase is typically 2–4 nmol/mg protein (2.5 nmol in this experiment). Thus, according to Simpson’s pool model, cholesterol sulfate is inhibiting the movement of cholesterol into the steroidogenic pool, rather than affecting its metabolic conversion.

Effect of Cholesterol Sulfate on the Metabolism of Endogenous Cholesterol in Adrenal Mitochondria—In our laboratory, adrenal mitochondria from ether-stressed rats typically contain 10–20 nmol of cholesterol per mg of protein, a range...
Fig. 2. Inhibition by cholesterol sulfate of the slow phase of pregnenolone production in rat adrenal mitochondria. Mitochondria were preincubated with cholesterol sulfate for 30 min prior to addition of 0.13 mM (final) cholesterol and initiation of the reaction with malate, as described in Fig. 1. Pregnenolone formation was measured by radioimmunoassay, as described under “Experimental Procedures.” Preincubations were carried out as follows: open squares, no additions; filled squares, 100 μM cholesterol sulfate; open triangles, 150 μM cholesterol sulfate.

Fig. 3. Cholesterol sulfate effect on the cleavage of endogenous cholesterol in mitochondria from ether-stressed rats. Cholesterol (filled symbols) and pregnenolone (open symbols) were determined by gas chromatography as under “Experimental Procedures.” Mitochondria were preincubated for 30 min with (triangles) or without (squares) cholesterol sulfate (27 μM), and the reaction was initiated as in Fig. 1.

Similar to values reported from other laboratories (9, 11). Upon addition of malate, part of this cholesterol is rapidly converted into pregnenolone, while the remainder is metabolized more slowly if at all. Fig. 3 shows that when no exogenous cholesterol is provided, slightly more than 2 nmol of pregnenolone per mg of protein is produced rapidly, a value which corresponds in rate and extent to the pregnenolone production seen above (Fig. 2) in the rapid phase when exogenous cholesterol is provided. It therefore can be concluded that the rapid phase in Fig. 2 is the metabolism of endogenous cholesterol which is in the steroidogenic pool in mitochondria following isolation. This interpretation is also supported by data in Fig. 1 which shows that exogenous radiolabeled cholesterol is metabolized linearly for over 30 min, without an initial rapid phase.

As shown in Fig. 3, cholesterol sulfate has no effect on the malate-induced depletion of cholesterol or on the corresponding increase in pregnenolone. Thus, activity data reveal that cholesterol sulfate does not exert its effect on cytochrome P-450scc or on the interaction of the enzyme with cholesterol in the steroidogenic pool.

To investigate this point more fully, we used aminogluthethimide-treated rats. In vivo pretreatment of rats with this cytochrome P-450scc inhibitor, followed by ether stress, increases both the quantity of mitochondrial cholesterol and the magnitude of the rapid phase of pregnenolone formation2 (38). The latter correlates with ar. increase in cholesterol in the inner mitochondria membrane (23). This pretreatment thus increases the quantity of cholesterol in this steroidogenic pool. As shown in Fig. 4 (compare with Fig. 3), aminogluthethimide caused an increase in cholesterol content from about 13 nmol/mg to about 50 nmol/mg mitochondrial protein, and increased by more than 10-fold the quantity of pregnenolone formed in the rapid phase. Cholesterol sulfate (either 27 μM [not shown]) or 150 μM, see Fig. 4) had no significant effect on either malate-induced cholesterol depletion or on pregnenolone generation. Thus, the metabolism of cholesterol in the steroidogenic pool is not affected by cholesterol sulfate.

Effect of Cholesterol Sulfate on Pregnenolone Production from 25-Hydroxycholesterol—To evaluate further whether cholesterol sulfate could be affecting the cleavage of exogenous cholesterol by inhibiting cytochrome P-450scc or its electron transport system, we utilized the alternate substrate 25-hydroxycholesterol. This compound binds to purified cytochrome P-450scc with an affinity similar to that of cholesterol (24, 35), but is considerably more water-soluble (39). Probably due to its increased solubility, it has more direct access to the enzyme than does cholesterol, thus bypassing the kinetic barrier to cholesterol utilization (40, 41). As shown in Fig. 5 (upper panel), cholesterol sulfate in concentrations which inhibited greater than 90% of the metabolism of radiolabeled cholesterol did not affect the cleavage of 25-hydroxycholesterol. The sparing of endogenous cholesterol (lower panel, Fig. 5; compare with lower panel, Fig. 3) indicates that under these conditions, 25-hydroxycholesterol is the preferred substrate and competes effectively for the utilization of endogenous cholesterol. These data support the conclusion that 25-hydroxycholesterol inhibits [3H]cholesterol utilization (see Ta-

2 Aminogluthethimide is lost during the isolation of mitochondria (23, 38), so that addition of reducing substrate allows active side chain cleavage of cholesterol to occur.
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Fig. 5. Cholesterol sulfate effect on the cleavage of exogenously added 25-hydroxycholesterol. Symbols and methods are as in Fig. 3, except that 25-hydroxycholesterol (50 μM) was added immediately prior to initiation of the reaction.

Fig. 6. Effect of cholesterol sulfate versus 25-hydroxycholesterol on the absorbance spectrum of adrenal mitochondrial cytochrome P-450. Mitochondria (0.25 mg/ml) from ether-stressed rats were split into sample and reference cuvettes and titrated with either cholesterol sulfate (upper panel) or 25-hydroxycholesterol (lower panel). An equivalent amount of solvent was added to the reference cuvette, prior to recording each difference spectrum. The change in absorbance at 420–390 nm was then quantified from the recorded spectra and plotted as a function of added steroid. In the upper panel, the 20α,22R-dihydroxycholesterol (1 μM) was added to induce a maximal high spin absorbance change (filled triangle). In the lower panel, the filled square represents the extrapolated maximal absorbance change obtained from an inverse plot of 25-hydroxycholesterol-induced absorbance changes.

ble I) by acting as a competitive substrate for cytochrome P-450<sub>inc</sub>, and indicate that it has ready access to the cytochrome. These data provide further evidence that cholesterol sulfate does not affect directly the activity of cytochrome P-450<sub>inc</sub>.

Effects of Cholesterol Sulfate and 25-Hydroxycholesterol on the Absorbance Spectrum of Mitochondrial Cytochrome P-450—The interaction of 25-hydroxycholesterol as well as the apparent lack of interaction of cholesterol sulfate with adrenal mitochondrial cytochrome P-450<sub>inc</sub> were investigated further using difference absorbance spectroscopy. In Fig. 6, a negative absorbance change represents conversion to the high spin absorbance spectrum. Cholesterol sulfate added in the concentration range that caused inhibition (see below) did not perturb significantly the spectrum of the mitochondrial cytochrome P-450. For comparison, addition to the sample cuvette of 1 μM 20α,22R-dihydroxycholesterol, a high spin-inducing steroid (24), produced the absorbance change indicated by the filled triangle.<sup>3</sup> This steroid was used as a control, since all 3β-modified cholesterol derivatives which interact with cytochrome P-450<sub>inc</sub> are known to produce exclusively the high spin spectrum (24).

In contrast, a 25-hydroxycholesterol-induced absorbance change was readily demonstrated (lower panel, Fig. 6) and occurred at nearly the same half-maximal concentration as that for inhibition by this steroid of metabolism of tritiated cholesterol (12 and 8 μM, respectively, see below). Complete binding of 25-hydroxycholesterol produces only about 25–30% conversion to the high spin state using the purified enzyme system (24, 43), thus accounting for the smaller maximal absorbance change (filled squares, Fig. 6) compared with that produced by 20α,22R-dihydroxycholesterol (compare upper and lower panels in Fig. 6).

Effect of Mitochondrial Sonication on the Inhibition by Cholesterol Sulfate of Cholesterol Side Chain Cleavage—Experiments described above show that cholesterol sulfate inhibits the side chain cleavage of exogenous cholesterol at a site other than cytochrome P-450<sub>inc</sub> and prior to entry of cholesterol into the steroidogenic pool. According to a working hypothesis, metabolism requires that the poorly water-soluble cholesterol (which presumably first encounters the outer mitochondrial membrane) be translocated across the aqueous intermembrane space to the inner membrane. Thus, a likely locus of cholesterol sulfate inhibition is an intermembrane cholesterol translocation system. Since in this model the aqueous intermembrane space represents the barrier to cholesterol utilization, disruption of this barrier should eliminate or reduce the efficacy of the translocation-specific inhibitor.

Fig. 7 demonstrates the concentration dependence for inhibition of side chain cleavage of radiolabeled cholesterol in intact versus sonicated mitochondria. Sonic disruption typically increased the activity by about 50%, consistent with a loss of the barrier to cholesterol utilization; data have been normalized in Fig. 7 to the percent of control (either intact or sonicated) to facilitate comparison. Sonication (water bath-type sonicator) reduced the inhibition at 44 μM cholesterol sulfate from 100 to 28%. Increasing the cholesterol sulfate up to 200 μM in this experiment (not shown) caused little further inhibition. (The partial inhibition seen in sonicated mitochondria showed the same half-maximal inhibitory concentration as that for the complete inhibition in intact mitochondria, suggesting that the residual inhibition may have been due to the presence of residual transport-dependent structures which are resistant to disruption. Longer periods of sonication or sonications with a more powerful probe-type sonicator did not further affect this partial inhibition.)

The relief of almost

3 Not shown in Fig. 6 are controls in which pregnenolone was added to the reference cuvette following addition of 20α,22R-dihydroxycholesterol to the sample in order to produce the maximal possible high spin conversion of cytochrome P-450<sub>inc</sub>. This procedure allows calculation of the initial content of high spin hemoprotein in this mitochondrial preparation; an initial content of 11% high spin was revealed. Since adrenal mitochondria contain approximately 1 nmol of cytochrome P-450<sub>inc</sub> per mg of protein (42), only about 0.1 nmol is initially present in the high spin form. If high spin enzyme is equated with substrate-bound enzyme (6), then the initial burst of pregnenolone formation cannot be accounted for by the presence of an enzyme-substrate complex.

4 3β-Methyl and -ethyl (but not larger) ethers of cholesterol bind relatively tightly to the purified enzyme, while cholesterol esters bind poorly, if at all (24). Cholesterol sulfate does not cause a spectral change indicative of binding to the purified enzyme, but causes some spectral changes which apparently correlate with denaturation of the cytochrome, as indicated by an increased conversion to the P-420 form of the hemoprotein (B. H. Heyl and J. D. Lambeth, unpublished results).
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70% of the inhibition by sonication provides evidence that the site of inhibition by cholesterol sulfate is an intramitochondrial cholesterol translocation system.

In contrast, the inhibition by 25-hydroxycholesterol which was shown above to inhibit due to its interaction with mitochondrial cytochrome P-450 was not relieved by sonication (see Fig. 8). In fact, sonication appeared to enhance slightly its inhibitory potency. Thus, inhibition by cholesterol sulfate but not by 25-hydroxycholesterol depends upon the intact structure of the mitochondrion.

**Fate of Added Cholesterol Sulfate**—To ensure that it was cholesterol sulfate and not a side chain cleaved or desulfated metabolite which was the active inhibitor, we investigated the possible conversion of added tritiated cholesterol sulfate into pregnenolone sulfate, cholesterol, and pregnenolone. It has been reported previously that under some circumstances, cholesterol sulfate can be converted into pregnenolone sulfate (30, 44-46). Side chain cleavage of cholesterol sulfate was first tested using the purified beef cytochrome P-450<sub>450<sub> system. As shown in Fig. 9, using the highly purified enzyme, no conversion to either pregnenolone sulfate or pregnenolone could be demonstrated at times up to 80 min, whereas cleavage of cholesterol was almost 80% complete at 30 min.

FIG. 7. Effect of sonication on the inhibition of cholesterol side chain cleavage by cholesterol sulfate. The rate of side chain cleavage was determined from kinetic plots as shown in Fig. 1, as a function of cholesterol sulfate concentration in either intact (open squares) or sonicated (filled squares) mitochondria. The same mitochondrial preparation was used.

FIG. 8. Effect of sonication on the inhibition of cholesterol side chain cleavage by 25-hydroxycholesterol. Side chain cleavage rates were determined as in Fig. 1 at various 25-hydroxycholesterol concentrations in either intact (open squares) or sonicated (filled squares) mitochondria.

FIG. 9. Side chain cleavage of cholesterol versus cholesterol sulfate by purified beef cytochrome P-450<sub>450<sub>. Side chain cleavage of either [3H]cholesterol (open squares) or [3H]cholesterol sulfate (filled squares), both at 44 µM, by purified cytochrome P-450<sub>450<sub> was quantified as described under “Experimental Procedures.”

FIG. 10. Side chain cleavage of cholesterol sulfate by intact and sonicated rat adrenal mitochondria. Cleavage to pregnenolone sulfate was quantified as described under “Experimental Procedures” using either intact (filled squares) or sonicated (open squares) adrenal mitochondria obtained from ether-stressed rats. For sonicated mitochondria, adrenodoxin reductase, adrenodoxin, and an NADPH-reducing system plus NADPH were included, as detailed under “Experimental Procedures.”

In isolated mitochondria, no cholesterol sulfatase activity could be demonstrated at incubation times up to 60 min, since neither free cholesterol nor free pregnenolone was detected by HPLC (see “Experimental Procedures”). In contrast to the situation with the isolated enzyme, an inhibitory concentration of cholesterol sulfate was slowly (but reproducibly) converted by adrenal mitochondria (both intact and sonicated) into pregnenolone sulfate (see Fig. 10), as has been reported previously (30, 44-46). Sonication increased the activity in cholesterol sulfate side chain cleavage by almost 2-fold at 40 min, consistent with the observations of Lieberman and colleagues (30, 44, 45) in beef mitochondria. At 30 min, approx...
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The present studies have provided evidence for a cholesterol sulfate-inhibitable translocation system that facilitates cholesterol movement into the steroidogenic pool. As described above, once cholesterol is in this pool the inhibitor has no effect. In addition, hallmark of any inhibitor of a transport system (more commonly transmembrane transport of polar molecules) is that removal or destruction of the barrier to transport (e.g. a membrane) should eliminate the effect of the inhibitor. In the present case, the translocated molecule is hydrophobic, and the barrier is the aqueous intermembrane space. Thus, destruction by sonication of the intact mitochondrial structure results in significant relief of inhibition, as predicted for a transport-specific effect. Since ACTH apparently stimulates side chain cleavage by promoting redistribution of cholesterol into the steroidogenic pool, the simplest interpretation is that the cholesterol sulfate-inhibitable translocation system is identical to that which is activated by ACTH. We suggest that the Steroidogenic Activator Peptide of Pedersen and Brownie (12) mediates this activation via effects on this translocation system.

Possible Physiological Relevance of Cholesterol Sulfate Inhibition—Several physiological roles for cholesterol sulfate have been postulated. First, due to its increased solubility compared with cholesterol it may function in the transport of cholesterol between tissues (33). Second, it is a component of biological membranes and stabilizes the red cell membrane to hypotonic lysis (54, 55). Third, as supported by the studies of Lieberman and colleagues (see Footnote 4), it can serve as a precursor of sulfated steroid hormones. In addition, this steroid is found in high levels in brain nerve endings (53), and its level correlates with developmental stage. Also, levels in certain cell types increase dramatically upon differentiation (56).

The present studies suggest that cholesterol sulfate may have an additional function as a regulator of steroid hormone production in adrenal cortex. Levels of cholesterol sulfate in beef adrenals are reported to be a minimum of 1.5 mg/kg wet weight of tissue (57). Assuming an equal distribution, one can calculate a minimum of 3 μM cholesterol sulfate in adrenal tissue, a value slightly lower than the Km of 6 μM for inhibition, but well within a range which might affect steroidogenic activity. It is also possible that cholesterol sulfate levels change, depending upon the physiological state of the tissue. It is intriguing in this context that chronic in vivo ACTH treatment is reported to activate a 3β,Δ5-specific steroid sulfatase activity in rat adrenals (58). Although the physiological relevance of possible modulation of steroidogenic activity by cholesterol sulfate is not yet understood, a recent study by Williams and co-workers (59) provides precedent for regulation of steroid pathways by this molecule: cholesterol sulfate was found to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and sterol biosynthesis in cultured fibroblasts, and the authors suggested cholesterol sulfate as an endogenous regulator of cholesterol biosynthesis. Thus, as more is learned about the physiological modulation of cholesterol sulfate levels, a new general role may emerge for cholesterol sulfate as a regulator of cholesterol biosynthesis and its subsequent metabolic conversion to steroid hormones.

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REFERENCES

2. Lambeth, J. D. (1985) in Phospholipids and Cellular Regulation

imimately 0.6 nmol of cholesterol sulfate per mg of protein was cleaved to pregnenolone sulfate, compared with 15–20 nmol when cholesterol was used as substrate (see Fig. 2). No other radiolabeled peaks were detected. Conversion thus produces less than 1 μM pregnenolone sulfate in the incubation, a concentration which is not inhibitory (see Table I). Thus, cholesterol sulfate rather than a metabolite is the inhibitory species.

**DISCUSSION**

**Cholesterol Sulfate Inhibits Side Chain Cleavage at a Site Distinct from Cytochrome P-450**—The present studies have shown that in whole mitochondria, cholesterol sulfate inhibits the side chain cleavage of exogenously added cholesterol, but at a site distinct from the side chain cleavage enzyme. That cholesterol sulfate does not interact with the cytochrome is supported by both absorbance spectroscopy (showing a failure of cholesterol sulfate but not several known substrates to perturb the P-450 spectrum) and activity studies (showing a failure of cholesterol sulfate to inhibit either the metabolism of endogenous cholesterol or of the alternate substrate, 25-hydroxycholesterol). Since the metabolism of these substrates requires the activity of both cytochrome P-450α and its electron transport chain, the locus of inhibition is not the side chain cleavage enzyme system. Nevertheless, the structural specificity for inhibition indicates that the inhibited system is likely to be protein in nature.

The failure of cholesterol sulfate to interact spectroscopically with or be metabolized by purified beef cytochrome P-450 further supports a locus of inhibition other than this enzyme. These data need not contradict the data of Greenfield and co-workers (51) who reported cholesterol sulfate binding and side chain cleavage activity of a purified cytochrome P-450 preparation. Their preparation method was quite different from ours, and in their hands, their cytochrome had dissimilar steroid binding and chromatographic properties compared with enzyme prepared in their laboratory according to our methodology. These workers suggested that their preparation may contain a unique side chain cleavage enzyme with more specificity for cholesterol esters. Although the issue is not yet resolved, this interpretation is supported by earlier kinetic and inhibition studies in beef mitochondria (48, 49) and in a partially purified beef cytochrome P-450 preparation (52), which implicated two side chain cleavage systems, one specific for cholesterol, and the other for cholesterol esters. Based upon the low rate of cholesterol sulfate side chain cleavage in rat adrenal mitochondria, such a second enzyme system would appear to be quantitatively minor. Its presence cannot account for the inhibition of cholesterol side chain cleavage by cholesterol sulfate (e.g. by "stealing" reducing equivalents from the electron transport system) since the cleavage of both 25-hydroxycholesterol and endogenous cholesterol is unaffected.

**Cholesterol Sulfate Inhibition Appears to be Specific for a Cholesterol Translocation System**—The poor water solubility of cholesterol together with the unique structure of the mitochondrial double membrane with an intervening aqueous intermembrane space are likely to provide a barrier to the movement of cholesterol into the inner membrane of most mitochondria, wherein cholesterol levels are known to be quite low. The existence in adrenal mitochondria of a barrier to cholesterol movement was suggested from earlier studies, summarized above. Further support was obtained from the studies of Stevens et al. (28) that showed that in mitoplast preparations (in which the outer mitochondrial membrane was removed) cholesterol could be readily metabolized, regardless of the prior activation state of the mitochondria.
Evidence for an Intramitochondrial Cholesterol Translocator