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THE SIDE-CHAIN CLEAVAGE OF CHOLESTEROL SULFATE—II. THE EFFECT OF PHOSPHOLIPIDS ON THE OXIDATION OF THE STEROL SULFATE BY INNER MITOCHONDRIAL MEMBRANES AND BY A RECONSTITUTED CHOLESTEROL DESMOLASE SYSTEM

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Summary—This study compares the side-chain cleavage of aqueous suspensions of cholesterol sulfate with the side-chain cleavage of cholesterol sulfate which is incorporated into phospholipid vesicles. Three different cholesterol desmolase systems are examined: (1) the membrane-bound cholesterol side-chain cleavage system present in inner mitochondrial membranes isolated from bovine adrenal mitochondria; (2) a soluble, lipid-depleted, reconstituted side-chain cleavage system prepared from cytochrome P-450scc, adrenodoxin and adrenodoxin reductase; (3) a membrane associated side-chain cleavage system prepared by adding phospholipid vesicles, prepared from adrenal mitochondrial, to the reconstituted system.

Soluble cholesterol sulfate, in low concentration, is a good substrate for the lipid-depleted reconstituted side chain cleavage system. However, at concentrations above 2 μM, in the absence of phospholipids, the sterol sulfate appears to bind at a non-productive site on cytochrome P-450scc which leads to substrate inhibition. Phospholipids, while inhibiting the binding of cholesterol sulfate to the cytochrome, also appear to prevent non-productive binding of the sterol sulfate to the cytochrome. Thus the addition of phospholipids to the lipid-depleted enzyme system leads to an activation of side-chain cleavage of high concentrations of the sterol sulfate.

Soluble cholesterol sulfate is a good substrate for both the native and reconstituted membrane-bound systems and no substrate inhibition is observed when the membrane bound enzyme systems are employed in the assay of side-chain activity. However, the cleavage of cholesterol sulfate, which is incorporated into phospholipid vesicles, by both membrane bound enzyme systems appears to be competitively inhibited by the phospholipids of the vesicles.

The results of this study suggest that the regulation of the side-chain cleavage of cholesterol sulfate may be entirely different than the regulation of the side-chain cleavage of cholesterol, if cholesterol sulfate exists intracellularly as a soluble non-complexed substrate. If, on the other hand, cholesterol sulfate is present in the cell in lipid droplets as a complex with phospholipids, its metabolism may be under the same constraints as the side-chain cleavage of cholesterol.

INTRODUCTION

Steroid sulfates are abundant metabolites of testicular and fetal adrenal tissues [1, 2]. In addition, dehydroepiandrosterone sulfate is present in large quantities in adult human blood [3]. Despite their abundance, little is known about the biosynthesis or function of the steroid sulfates. In 1964, Lieberman and coworkers showed that cholesterol sulfate could be converted *in vivo* in humans into dehydroepiandrosterone sulfate without loss of the sulfate moiety. This study demonstrated that cholesterol sulfate is a substrate for the cholesterol side-chain cleavage system [4]. Cholesterol sulfate has also been shown *in vitro* to be a substrate for side-chain cleavage by enzyme systems present in rat and bovine mitochondria [5]. Although cholesterol sulfate accounts for only a small part of the total cholesterol present in bovine adrenal glands, its absolute concentration is non-trivial, 1.5 mg/kg wet weight [6]. Thus substantial amounts are available as a potential substrate for cholesterol side-chain cleavage in bovine adrenal tissue. Nothing is known about the intracellular compartmentalization of cholesterol sulfate. Cholesterol sulfate is generally considered to be a soluble substance and it was originally extracted from bovine adrenal tissue with ammonium hydroxide. However, the steroid sulfate can be incorporated into phospholipid vesicles and the complexed cholesterol sulfate can then be extracted into organic solvents such as methylene chloride. Cholesterol sulfate is

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Abbreviations and trivial names: Cholesterol, 5-cholesten-3β-ol; cholesterol sulfate, 5-cholesten-3β-yl sulfate; pregnenolone, 3-hydroxy-5-pregnene-20-one; Tris, tris-(hydroxymethyl) methylamine; cytochrome P-450scc, P-450scc, P-450, the cytochrome which catalyzes the side-chain cleavage of cholesterol to yield pregnenolone and isocaproaldehyde; Tween 80, polyethylenesorbitanmonooleate; DTT, dithiothreitol; ICA, isocaproaldehyde.

found in the low density lipoprotein fraction of the blood [7]. It is thus possible, that in the cell the cholesterol sulfate is associated with the membrane phospholipids.

This paper examines the effect of incorporating cholesterol sulfate into phospholipid vesicles on the rate of the side-chain cleavage of the sterol sulfate by cholesterol desmolase. The vesicles were prepared from phospholipids which were isolated from mitochondria of the adrenal cortex. Three different enzyme systems were utilized in this study: 1. The membrane bound, relatively intact, side-chain cleavage system that is present in inner mitochondrial membranes which are isolated from bovine adrenal tissues; 2. A soluble lipid-depleted reconstituted side-chain cleavage system prepared with purified cytochrome P-450scc, adrenodoxin and adrenodoxin reductase; 3. A membrane-bound reconstituted side-chain cleavage system which was prepared by incubating the reconstituted side-chain cleavage enzyme system with preformed phospholipid vesicles prepared from adrenal cortex mitochondria. The effects of the addition of phospholipids on the activity of the enzyme systems are contrasted.

EXPERIMENTAL

Dithiothreitol (DTT), ethylenediaminetetraacetate disodium salt (EDTA), Tween 80, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADP were obtained from Sigma Chemical Co. (St Louis, MO). NADPH was obtained from Boehringer Mannheim (West Germany). Glycerol was spectroquality grade from Fisher Scientific Co. (Fairlawn, NJ). Ultrapure Tris buffer and sucrose were obtained from Swartz-Mann (Spring Valley, NY). Cholesterol labeled at the C-26 position with ^{14}C was obtained from New England Nuclear (Boston, MA) and purified as described by Gasparini[8]. Labeled cholesterol sulfate was also prepared and purified as described by Gasparini[8].

Preparation of the cholesterol side-chain cleavage systems

Inner mitochondrial membranes. Fresh bovine adrenals were obtained from a slaughterhouse and chilled on ice until the mitochondria could be prepared (1–3 h). The medulla were excised and the cortical tissue was removed from the tissue membranes by gentle scraping with a scalpel. The minced cortical tissue was homogenized in 0.25 M sucrose, 0.01 M Tris–HCl, 1 mM DTT and 1 mM EDTA, pH 7.4 (3 ml/g tissue) for 1 min at high speed and 1 min at low speed in an Osterizer Blender. The cell debris was removed by centrifugation at 600 *g* for 20 min. The mitochondria were collected by centrifugation at 10,000 *g* for 30 min. Inner mitochondria membranes were prepared by swelling the mitochondria in a hypotonic solution and collecting the inner membrane pellet by centrifuging the membranes through a Ficoll gradient as described by Privalle *et al.*[9]. The mitochondria membranes were suspended in 0.25 M sucrose, 0.01 M Tris–HCl, 1 mM EDTA, 1 mM DTT, 20% glycerol, pH 7.4 and the suspended membranes were stored in 1 ml aliquots at −70°C for several days until needed. The membranes were defrosted and immediately used for assay. Unused defrosted membranes were discarded.

Reconstituted side-chain cleavage system. Cytochrome P-450scc was isolated from bovine adrenal cortex by a modification of the method of Greenfield *et al.*[10] as described previously [11]. As isolated the heme of the preparation was mainly in the low spin configuration. The ratio of absorbance at 280–416 nm was 1.2:1. The preparation had a low endogenous phospholipid content of 1–2 nmol/nmol P-450 and the heme to protein ratio was 14 nmol/mg. Adrenodoxin was prepared by the method of Suhara *et al.*[12] or purchased from Sciogen, Chicago, IL, and adrenodoxin reductase was prepared by the method of Lambeth and Kamin[13].

Characterization of the side-chain cleavage systems

Protein contents were estimated by the microbiuret procedure of Goa[14] or the procedure of Bradford[15]. Cytochrome P-450 was determined by the method of Omura and Sato[16].

Preparation of phospholipids

Phospholipids were extracted from intact mitochondria isolated from bovine adrenal cortex by the method of Folch *et al.*[17] and purified by chromatography on silica gel as described by Rouser *et al.*[18]. Phospholipids were quantified by the method of Chalvardjian and Rudnicki[19].

Assays of enzyme activity

Assays of enzyme activity were performed by following the release of ^{14}C-labeled isocaproaldehyde from ^{14}C-26 cholesterol or cholesterol sulfate by the method of Hochberg[5] with the following modifications: The substrate concentrations used in the assays ranged from 0.5 to 7 μM. The assay mixtures were preincubated at 37°C for 3 min and the assays were initiated by addition of NADPH containing an NADPH regeneration system. The final concentrations of NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase in the incubation mixture were 1 mM, 10 mM and 1.25 units/ml respectively.

When the inner mitochondrial membranes were employed as the source of the side-chain cleavage enzyme the assay mixture contained between 0.2–2.0 nmol/ml of P-450. No additional adrenodoxin or adrenodoxin reductase was added to the incubations. Cholesterol sulfate and cholesterol were assayed in the absence of phospholipids, by simply suspending the substrates in 0.1 M Tris–HCl, pH 7.4 by sonification. In addition, the substrates were incorporated into phospholipid vesicles by evaporating

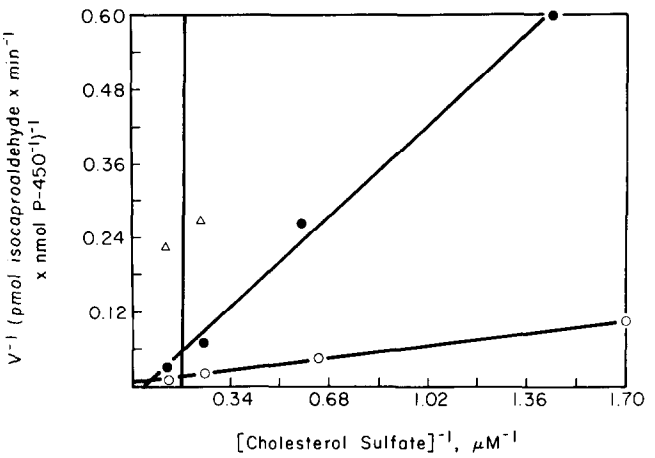

Fig. 1. The effect of incorporating cholesterol sulfate into vesicles prepared with adrenal mitochondrial phospholipids on its side-chain cleavage by inner mitochondrial membranes from bovine adrenal cortex. P-450 = 0.2 μM. (○ ○ ○ ○) no phospholipid, (● ● ● ●) phospholipid = 2 μM (△ △) phospholipid = 20 μM.

mixtures of the substrates and phospholipids in chloroform to dryness under nitrogen, adding 0.1 M Tris–HCl, pH 7.4 and sonifying in a bath sonifier (Branson model B-32) at 150 W power until the solutions cleared (approx 15 min). The final concentrations of phospholipid used in these experiments ranged from 2–20 nmol/ml.

When the reconstituted side-chain cleavage system was used, assay mixtures contained 0.01 μM P-450scc, 0.1 μM adrenodoxin reductase and 1.0 μM adrenodoxin in 0.1 M Tris–HCl, 0.2 mM dithiothreitol, 0.1 mM $MgCl_2$ pH 7.4. These concentrations of P-450, adrenodoxin and adrenodoxin reductase are the same as those employed by Wang and Kimura[20] in their "standard" assay of cholesterol side-chain cleavage activity.

The effects of the addition of phospholipids on the activity of the reconstituted cholesterol side-chain cleavage system were tested using several different experimental protocols. First, the soluble "lipid-free" reconstituted enzyme system was used to determine the rates of cleavage of both substrates which were simply suspended in aqueous solution by sonication. Second, the reconstituted enzyme system was incorporated into phospholipid vesicles, at ratios of phospholipid:P-450 of 100:1 and 200:1, by preincubating the enzyme with performed phospholipid vesicles, as described by Seybert *et al.*[21]. The membrane-bound enzyme systems were used to assay the rate of cleavage of the aqueous suspensions of cholesterol and cholesterol sulfate. Third, the substrates were incorporated into phospholipid vesicles (again at ratios of phospholipid:P-450 of 100:1 and 200:1) and the membrane-bound cholesterol or cholesterol sulfate was used as a substrate for the soluble, membrane-free, enzyme system. Finally, the enzyme and the two substrates were each associated with their own vesicles, and the membrane-bound cholesterol or cholesterol sulfate was used as a substrate for the membrane-bound enzyme system. The phospholipid:P-450 ratio in the enzyme and substrate vesicles were both 100:1, i.e. phospholipid, 1 μM, was added to both the enzyme and the substrates.

RESULTS

The side-chain cleavage of cholesterol and cholesterol sulfate by inner mitochondrial membranes

Cholesterol sulfate, in aqueous suspension, was a good substrate for the side-chain cleavage enzyme present in the inner mitochondrial membranes. The apparent K_m of cleavage of cholesterol sulfate was a function of the concentration of the inner membranes used in the assay mixture; the apparent K_m value for the cleavage of cholesterol sulfate increased as the membrane concentration increased probably because the oxidation of CS was competitively inhibited by both the phospholipids and endogenous cholesterol present in the inner mitochondrial membranes. When the P-450 concentration used in the assay was equal to 0.2 μM, the K_m of cleavage of cholesterol sulfate by the mitochondrial membranes was 8 ± 4 μM and the maximal rate of cleavage was 0.14 ± 0.07 nmol cholesterol sulfate cleaved/min·nmole cytochrome P-450scc. When the P-450 concentration was increased to 2 μM the K_m of cleavage of cholesterol sulfate was increased to 27 ± 11 μM, however the maximal rate of cleavage was, within experimental error, unchanged, and was equal to 0.10 ± 0.04 nmol/min·nmol.

The effects of incorporating the sterol sulfate into phospholipid vesicles on its kinetics of cleavage by the side-chain cleavage system present in the inner mitochondrial membranes are illustrated in Figs 1 and 2. Figure 1 illustrates the data obtained using 0.2 μM membrane bound cytochrome P-450 and Fig. 2 illustrates the data obtained using 2.0 μM membrane bound cytochrome P-450. When vesicles

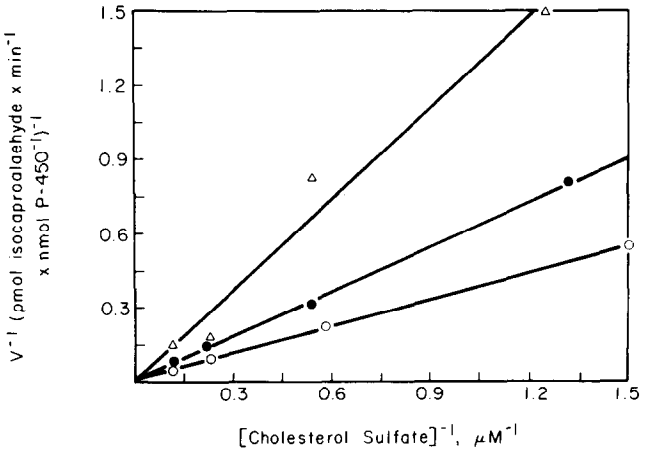

Fig. 2. The effect of incorporating cholesterol sulfate into vesicles prepared with adrenal mitochondrial phospholipids on its side-chain cleavage by inner mitochondrial membranes from bovine adrenal cortex. P-450 = 2 μM. (○ ○ ○) no phospholipid, (● ● ●) phospholipid = 2 μM, (△ △ △) phospholipid = 20 μM.

containing mixtures of phospholipids and cholesterol sulfate were prepared and the cholesterol sulfate that was incorporated into the vesicles was used as the substrate for the side-chain cleavage system present in the inner mitochondrial membranes, the side-chain cleavage of the sterol sulfate was greatly inhibited. The degree of inhibition observed was a function of the concentration of the inner mitochondrial membranes used in the assay. The lower concentration of membranes, containing 0.2 μM P-450, showed almost no cleavage of cholesterol sulfate when the sterol sulfate was incorporated into the phospholipid vesicles. The observed rates of cleavage of cholesterol sulfate were too low to accurately determine the K_m values for the cleavage of the cholesterol sulfate which was suspended in the vesicles and the y-axis intercepts of Lineweaver–Burk plots [22] of the data were negative. The observed rate of cleavage of the highest concentration of aqueous cholesterol sulfate that was used in the assay, 7 μM, by the inner membrane at a concentration of 0.2 μM P-450 was 0.063 ± 0.03 nmol cholesterol sulfate cleaved/min·nmol P-450. When vesicles were prepared with 7 μM cholesterol sulfate and 2 μM phospholipid the rate of cleavage dropped to 0.03 ± 0.02 nmol/min·nmol and when vesicles were prepared with 7 μM cholesterol sulfate and 20 μM phospholipid the rate of cleavage of the cholesterol sulfate dropped to 0.003 ± 0.002 nmol/min·nmol P-450. When the solutions contained 2.0 μM membrane bound P-450, incorporation of cholesterol sulfate into phospholipid membranes appeared to lead to competitive inhibition of the cleavage of the cholesterol sulfate by the membrane lipids. The apparent K_i of inhibition of the mitochondrial phospholipids for the cleavage of the sterol sulfate was 5 ± 2 μM.

The simple addition of preformed phospholipid vesicles to the mitochondrial membranes before they were incubated with aqueous suspensions of cholesterol sulfate did not inhibit the side-chain cleavage of the sterol sulfate.

Cholesterol, while it can be suspended in Tris–HCl to a concentration of 6 μM (as measured by counting an aliquot of the suspension) shows a maximal rate of cleavage of only 0.005 mole/min·mol (approx $\frac{1}{20}$ the rate of oxidation of cholesterol sulfate. When the cholesterol is suspended in phospholipid vesicles (2 μM) its initial rate of oxidation is decreased, but by 9 min its rate of oxidation is increased approx 20% compared to cholesterol which is suspended in buffer by sonication alone. The rate of cleavage of cholesterol, which is incorporated into the vesicles, increases as a function of time. This result suggests that the phospholipids serve to solubilize the cholesterol so it becomes available to the side-chain cleavage enzyme but that the rate of transfer of cholesterol from the vesicle to the active site of the membrane bound side-chain cleavage system is slow. The cleavage of cholesterol is completely inhibited when the cholesterol is incorporated into phospholipid vesicles prepared with 20 μM phospholipid.

The cleavage of cholesterol sulfate by reconstituted side-chain cleavage systems: comparison with the side-chain cleavage of cholesterol

The effects of phospholipids on the kinetics of side-chain cleavage of cholesterol and cholesterol sulfate by reconstituted side-chain cleavage enzyme systems are summarized in Table 1. Phospholipid addition to the reconstituted side-chain cleavage system had very different effects on the rates of cleavage of cholesterol and cholesterol sulfate. Lineweaver–Burk plots [22] of the rate of cleavage of cholesterol and cholesterol sulfate, which are suspended in phospholipid vesicles, are illustrated in Figs 3 and 4 respectively.

The side-chain cleavage of cholesterol. Cholesterol, in the absence of phospholipids or detergents, is very

Table 1. The effect of phospholipids on the side-chain cleavage of cholesterol and cholesterol sulfate

Experimental conditions*	Concn substrate-free phospholipid μM	Concn phospholipid containing substrate μM	K_m μM	V_{max} mol ICA/min mol P-450scc
	Substrate = Cholesterol, 0.5–7.0 μM			
A	0	0	0.9 ± 0.7	1.6 ± 0.6
B	1	0	0.7 ± 0.6	0.5 ± 0.4
B	2	0	1.2 ± 0.4	0.2 ± 0.1
C	0	1	3.7 ± 1.3	7.8 ± 1.9
C	0	2	15 ± 5	13 ± 3
D	1	1	7.2 ± 1.0	4.3 ± 1.2
	Substrate = Cholesterol sulfate, 0.5–7.0 μM			
A	0	0	0.6 ± 0.4	4.5 ± 3.0
B	1	0	1.6 ± 0.8	6.2 ± 3.3
B	2	0	4.4 ± 2.1	5.1 ± 2.0
C	0	1	1.7 ± 0.7	6.8 ± 2.9
C	0	2	4.1 ± 3.9	3.9 ± 2.0
D	1	1	1.8 ± 0.5	1.1 ± 0.3

*Experimental conditions. The reconstituted side-chain cleavage system contained 0.01 μM cytochrome P-450scc, 0.1 μM adrenodoxin reductase and 1.0 μM adrenodoxin. A: The reconstituted enzyme system was preincubated for 3 min at 37°C with substrates that were suspended in 0.1 M Tris–HCl by sonication. The reaction was initiated with NADPH. B: The reconstituted enzyme system was preincubated with the "empty" vesicles of adrenal mitochondrial phospholipids for 30 min at 4°C. The lipid-associated enzyme was then preincubated for an additional 3 min at 37°C with the substrates which were suspended in Tris–HCl by sonication. The reaction was initiated with NADPH. C: The reconstituted enzyme system was preincubated with phospholipid vesicles which contained the substrates for 30 min at 4°C. The membrane bound enzyme system was then preincubated for an additional 3 min at 37°C. The reaction was initiated with NADPH. D: The enzyme system was preincubated for 30 min at 4°C with the phospholipid vesicles which did not contain substrate. The membrane-bound enzyme system was then incubated for an additional 3 min at 37°C with the phospholipid vesicles which contained the substrate. The reaction was initiated with NADPH. ICA is isocaproaldehyde.

poorly soluble in aqueous solutions [24]. The maximum "concentration" of cholesterol which could be suspended in 0.1 M Tris–HCl buffer by sonication was approx 6 μM. Over the "concentration" range of 0.3–6 μM the K_m of cleavage of aqueous cholesterol by the reconstituted system was approx 1 μM and the maximal observed rate of cleavage was approx 1 nmol cholesterol cleaved/min·nmol P-450. When the reconstituted enzyme system was incorporated into phospholipid membranes which did not contain substrate, at either 100:1 or 200:1 ratios of phospholipid:P-450, the side-chain cleavage of the aqueous cholesterol was non-competitively inhibited by the phospholipids. The maximal rate of cleavage dropped to 0.5 nmol/min·nmol when the enzyme system contained a 100:1 ratio of phospholipid:P-450 and to 0.2 nmol/min·nmol when the enzyme system contained a 200:1 ratio of phospholipid:P-450.

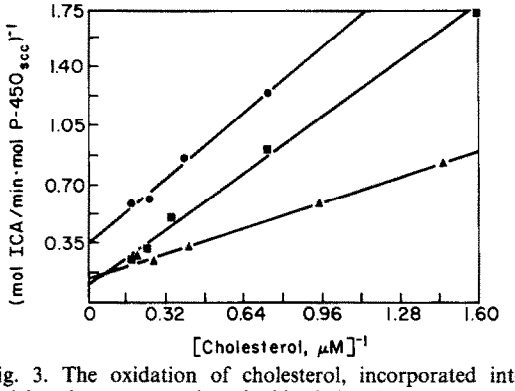

Fig. 3. The oxidation of cholesterol, incorporated into vesicles, by a reconstituted side-chain cleavage system. (▲ ▲ ▲) The enzyme was pre-incubated with vesicles prepared with 1 μM phospholipid and 0.5–7 μM cholesterol for ½ h at 4°C and for 3 min at 37°C. (■ ■ ■) The enzyme system was pre-incubated with vesicles prepared with 2 μM phospholipid and 0.5–7.0 μM cholesterol for ½ h at 4°C and 3 min at 37°C. (● ● ●) The enzyme system was pre-incubated with substrate-free vesicles prepared with 1 μM phospholipid for ½ h before the assay. The membrane-associated enzyme system was then incubated with substrate-containing vesicles prepared with 1 μM phospholipid and 0.5–7.0 μM cholesterol for 3 min at 37°C. The reconstituted enzyme system contained 0.01 μM cytochrome P-450scc, 1.0 μM adrenodoxin and 0.1 μM adrenodoxin reductase. All of the enzymatic reactions were initiated with NADPH. ICA is isocaproaldehyde.

When cholesterol was incorporated into the phospholipid vesicles, before they were added to the soluble membrane-free reconstituted system, the rate of side-chain cleavage of the lipid-bound cholesterol was greatly stimulated, compared to the rate of cleavage of the lipid-free substrate, although the K_m of cleavage of cholesterol was also increased. When the phospholipid:P-450 ratio was 100:1, the apparent K_m of cleavage of micelle-incorporated cholesterol was 4 ± 1 μM, but the V_{max} of cleavage was 8 ± 2 nmol/min·nmol. When the phospholipid:P-450 ratio was increased to 200:1, the K_m of cleavage of cholesterol by the membrane-bound enzyme system was further increased to 15 ± 5 μM. The maximal rate of cleavage of cholesterol, however, also appeared to be somewhat increased to 13 ± 3 nmol/min·nmol. However, the values of the K_d and V_{max} of

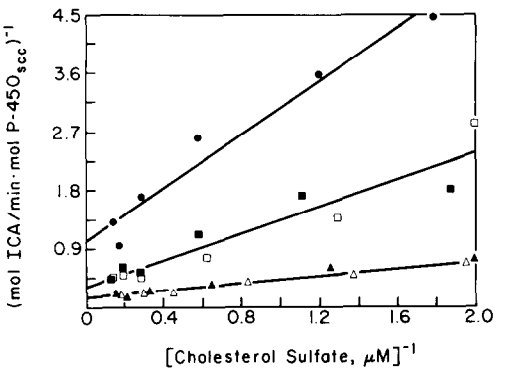

Fig. 4. The oxidation of cholesterol sulfate, suspended in aqueous solution by sonication, and cholesterol sulfate, incorporated into vesicles, by a reconstituted side-chain cleavage system. (▲ ▲ ▲) The enzyme system was pre-incubated with vesicles prepared with 1 μM phospholipid and 0.5–7.0 μM cholesterol sulfate for ½ h at 4°C and 3 min at 37°C. (△ △ △) The enzyme system was preincubated with substrate-free vesicles for ½ h at 4°C and the membrane-bound enzyme system was incubated with aqueous suspensions of cholesterol sulfate for 3 min at 37°C. (■ ■ ■) The enzyme system was pre-incubated with vesicles prepared with 2 μM phospholipid and 0.5–7 μM cholesterol sulfate for ½ h at 4°C and 3 min at 37°C. (□ □ □) The enzyme system was pre-incubated with substrate-free phospholipid for ½ h at 0°C and the membrane-bound enzyme was incubated with aqueous suspensions of cholesterol sulfate for 3 min at 37°C. (● ● ●) The enzyme system was incubated with substrate-free vesicles prepared with 1 μM phospholipid for ½ h a 4°C. The membrane-associated enzyme was incubated with vesicles prepared with 1 μM phospholipid and 0.5–7 μM cholesterol sulfate for 3 min at 37°C. The enzyme system contained 0.01 μM cytochrome P-450scc, 1.0 μM adrenodoxin and 0.1 μM adrenodoxin reductase. All of the enzymatic reactions were initiated with NADPH. ICA is isocaproaldehyde.

cleavage of cholesterol, obtained when the phospholipid:P-450 ratio was 200:1, are at best approximations of the true values as the maximal concentration of cholesterol, 7 μM, used in the assay of enzymatic activity was far from saturating under these assay conditions. Figure 3 illustrates Lineweaver–Burk plots [22]of the rate of cleavage of cholesterol as a function of cholesterol concentration at the two ratios of phospholipid to P-450 of 100:1 and 200:1.

Membrane-bound cholesterol was a good substrate for the soluble reconstituted side-chain cleavage enzyme system. However, the cleavage of cholesterol, which was suspended with phospholipids at a ratio of phospholipid to P-450scc of 100:1, was greatly inhibited if the cytochrome P-450 was preincubated with substrate free phospholipid vesicles, also at a ratio of 100:1 phospholipid:P-450, and the membrane-bound cholesterol was used as a substrate for the membrane-bound enzyme system. The apparent K_m of cleavage of membrane-bound cholesterol by the membrane-bound enzyme system was increased approx 2-fold relative to the K_m value when the soluble enzyme was used to cleave the membrane-bound cholesterol. Moreover the maximal rate of cleavage of cholesterol was decreased approx 2-fold. The inhibition of the cleavage of membrane-bound cholesterol by "empty" phospholipid vesicles is also illustrated in Fig. 3.

The side-chain cleavage of cholesterol sulfate. Cholesterol sulfate, unlike cholesterol, is easily suspended in aqueous solution by sonication in the absence of phospholipids. The maximal observed rate of cleavage of aqueous, membrane-free cholesterol sulfate was approx 4.5 nmol/min·nmol at a concentration of cholesterol sulfate of 1.5 μM. Above this concentration the cleavage of cholesterol sulfate appeared to be substrate inhibited and the reconstituted side-chain cleavage system showed no activity at all when the substrate concentration exceeded 5 μM.

When the enzyme system was assayed in the presence of two concentrations of adrenal mitochondrial phospholipids, 1 and 2 μM (ratios of phospholipid:P-450 of 100:1 and 200:1 respectively), the substrate inhibition was relieved. In contrast to the results obtained when cholesterol was the substrate, it did not matter whether the enzyme was preincubated with substrate free membranes and the membrane-bound enzyme system was used to assay the aqueous sterol sulfate, or if the substrate was incorporated into the phospholipid membranes and the aqueous lipid-free system was used to assay the membrane-bound substrate. As illustrated in Fig. 4, in each case the observed values of the K_m and V_{max} were within experimental error of one another. In the presence of a 100:1 ratio of phospholipid:P-450 the observed K_m of cleavage of cholesterol sulfate was approx 2 μM and the maximal rate cleavage of cholesterol sulfate was approx 7 mol/min·mol. When the phospholipid ratio was raised to 200:1 the K_m of cleavage of cholesterol sulfate was increased to approx 4 μM and the maximal rate of cleavage of cholesterol sulfate was decreased to 4 nmol/min·nmol compared to the respective values obtained when a 100:1 ratio of phospholipid:P-450 was used in the assay.

While it did not matter if either the substrate or the enzyme were incorporated into the phospholipid vesicles; i.e. the membrane-bound enzyme could utilize aqueous cholesterol sulfate as a substrate, and the aqueous enzyme system could utilize cholesterol sulfate which was incorporated into phospholipid vesicles as a substrate; the cleavage of cholesterol sulfate was greatly inhibited if the substrate and reconstituted systems were each incorporated into their own phospholipid vesicles. When the reconstituted side-chain cleavage system was incorporated into phospholipid vesicles, at ratio of phospholipid–heme of 100:1 the K_m of cleavage of the membrane-bound cholesterol sulfate (also containing a 100:1 ratio of phospholipid–heme) was approx 2 μM but the V_{max} of cleavage of cholesterol sulfate dropped to 1 nmol/min·nmol compared to 7 nmol/min·nmol when only the substrate or the P-450 was

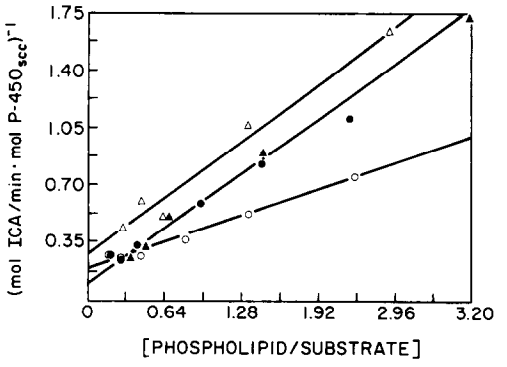

Fig. 5. The kinetics of cleavage of cholesterol and cholesterol sulfate, incorporated into phospholipid vesicles, by a reconstituted side-chain cleavage system. (● ● ●) substrate = cholesterol, phospholipid:P-450 = 100:1 (▲ ▲ ▲) substrate = cholesterol, phospholipid:P-450 = 200:1. (○ ○ ○) substrate = cholesterol sulfate, phospholipid:P-450 = 100:1 (△ △ △) substrate = cholesterol sulfate, phospholipid:P-450 = 200:1 The experimental conditions are given in Figs 3 and 4.

incorporated into the phospholipid vesicles. The effect of incorporating cytochrome P-450 into "empty" vesicles on the rate of side-chain cleavage of membrane-bound cholesterol sulfate is also illustrated in Fig. 4.

The rate of cleavage of cholesterol and cholesterol sulfate as a function of the ratio of phospholipid–substrate. Seybert *et al.*[21] have suggested that the rate of cleavage of cholesterol should not be expressed as a simple function of the total cholesterol concentration added to aqueous suspensions of cytochrome P-450, when the substrate is incorporated into phospholipid vesicles. Rather they suggest that the rate of cleavage of cholesterol should be expressed as a function of the ratio of phospholipid–cholesterol present in the vesicles containing the substrate. In order to determine the kinetic constants these authors plot 1/V (the rate of cleavage of cholesterol) as a function of the phospholipid–cholesterol ratio and they express the K_m of cleavage of cholesterol as the ratio of cholesterol–phospholipid which gives the $\frac{1}{2}$ maximal rate of cleavage of the membrane-bound sterol. Figure 5 compares the data obtained for the cleavage of cholesterol and cholesterol sulfate, which were incorporated into phospholipid vesicles containing 1 and 2 μM phospholipid, plotted according to the procedure of Seybert *et al.*[21]. The data obtained for the cleavage of cholesterol, at the two different phospholipid concentrations, did approximately fall on one straight line and the maximal rate of cleavage of cholesterol appeared to be almost independent of the phospholipid concentration. However, the data obtained for the cleavage of cholesterol sulfate did not fall on a single straight line. At the higher concentration of phospholipid the maximal rate of cleavage of cholesterol sulfate was significantly lower than it was at the lower concentration of phospholipid.

DISCUSSION

In this study the side-chain cleavage of aqueous cholesterol sulfate was compared to the side-chain cleavage of cholesterol sulfate which was suspended in phospholipid vesicles. Three different systems were used as sources of the side-chain cleavage enzyme: 1. A membrane bound enzyme system which was prepared by suspending inner mitochondrial membranes that were isolated from bovine adrenal cortex in Tris buffer; 2. A soluble, lipid-depleted, reconstituted side-chain cleavage system; 3. A membrane associated side-chain cleavage system that was prepared by incorporating the reconstituted system into phospholipid vesicles.

Cholesterol is very poorly soluble in aqueous solutions. When cholesterol is sonicated in water, only 0.01–0.02 μM of the sterol is actually solubilized. The remainder of the sterol which is suspended in solution by sonication probably is in the form of microcrystals of cholesterol monohydrate [23]. Cholesterol, in aqueous suspension, serves as a very poor substrate for the "intact" cholesterol side-chain cleavage system found in inner mitochondrial membranes which are isolated from bovine adrenal cortex and is also a poor substrate for the soluble membrane-free reconstituted cholesterol side-chain cleavage system prepared with purified cytochrome P-450scc, adrenodoxin and adrenodoxin reductase. A very low rate of cleavage of aqueous cholesterol by reconstituted side-chain cleavage systems has also been reported by Seybert *et al.*[21] and Hall *et al.*[24]. We have found, moreover, that if the purified reconstituted side-chain cleavage system is preincubated with phospholipid vesicles, the side-chain cleavage of aqueous suspensions of cholesterol is greatly inhibited by the phospholipids. Seybert *et al.*[21] have shown, on the other hand, that if cholesterol is incorporated into phospholipid vesicles it becomes a good substrate for soluble, reconstituted cholesterol side-chain cleavage systems. This result of Seybert *et al.*[21] has been confirmed in our present study. Our study also confirms their finding that the side-chain cleavage of membrane-bound cholesterol, by the soluble reconstituted enzyme system, is greatly inhibited if the enzyme is preincubated with phospholipid vesicles which do not contain substrate. Seybert *et al.*[21] have also observed that the rate of cleavage of cholesterol by membrane reconstituted side-chain cleavage enzyme systems apparently is a function of the phospholipid–cholesterol ratio within the phospholipid vesicles surrounding the enzymes, rather than a function of the total cholesterol concentration in the assay mixture. The results of this present study also confirm this observation, when cholesterol is the substrate.

Cholesterol sulfate, in contrast to free cholesterol, is easily suspended in aqueous solution by sonication and clear suspensions, containing 50 μM cholesterol sulfate, can be obtained. Aqueous cholesterol sulfate, moreover, is a good substrate for the "intact" choles-

terol side-chain cleavage system of inner mitochondrial membranes. The observed cleavage of the sterol sulfate by the membrane-bound enzyme systems indicates that the sterol sulfate can penetrate the phospholipid membranes. Cholesterol sulfate is also a good substrate for the soluble reconstituted side-chain cleavage system prepared with cytochrome P-450scc, 0.01 μM, adrenodoxin, 1 μM, and adrenodoxin reductase, 0.1 μM. However the oxidation of cholesterol sulfate is substrate inhibited by high concentration of the sterol sulfate. The addition of adrenal phospholipids prevents the substrate inhibition noted when high concentrations of cholesterol sulfate are used in the assay. However, in contrast to the case when cholesterol is the substrate, the maximal rate of cleavage of cholesterol sulfate is not stimulated when cholesterol sulfate is incorporated into phospholipid vesicles. Moreover, unlike the case when cholesterol is the substrate, the V_{max} of cleavage of cholesterol sulfate decreases when the phospholipid concentration in the assay is increased. Thus, unlike cholesterol, the rate of cleavage of cholesterol sulfate is not a function of only the phospholipid–substrate ratio used in the assay of side-chain cleavage activity, but the rate of cleavage was also a function of the phospholipid to P-450 ratio. Moreover, also in contrast to the case when cholesterol is used as the substrate, the order of addition of cholesterol sulfate and the phospholipid vesicles to cytochrome P-450scc has no effect on the enzymatic activity of the reconstituted side-chain cleavage system. Cholesterol sulfate, phospholipids and cytochrome P-450 appear to be in thermodynamic equilbrium under the conditions used in the assay of side-chain cleavage activity.

Membrane-bound cholesterol sulfate is a good substrate for the lipid-free enzyme system, and soluble cholesterol sulfate is a good substrate for the side-chain cleavage system which is incorporated into phospholipid membranes. However, when cholesterol sulfate is incorporated into phospholipid vesicles, and the side-chain cleavage enzyme system is also incorporated into its own substrate-free vesicles, the cleavage of cholesterol sulfate is very low. The low rate of cleavage of the membrane-bound cholesterol sulfate that is observed when the membrane bound enzyme system is employed in the assay. suggests that the rate of transfer of cholesterol sulfate between phospholipid vesicles is slow compared to the rate of oxidation of the substrate by the enzyme system.

The results of this study suggest that if cholesterol sulfate is present in the cell as a soluble, lipid-free substrate, it may serve as a substrate for the side-chain cleavage system which is present in the inner mitochondrial membrane. In contrast to cholesterol, no mechanism of activation may be necessary to transport the soluble cholesterol sulfate to the membrane bound enzyme, as aqueous suspensions of the sterol sulfate appear to be able to interact directly with the membrane bound side-chain cleavage system even in the absence of any detergents. The regulation of the side-chain cleavage of cholesterol sulfate if it exists *in vivo* as a soluble substrate could thus be entirely different from the regulation of the side-chain cleavage of cholesterol. On the other hand, if cholesterol sulfate is present in the cell in lipid droplets associated with phospholipids, its cleavage may be under the same constraints as the side-chain cleavage of cholesterol, i.e. some mechanisms would be necessary to facilitate the transport of cholesterol sulfate from its site of storage in lipid droplets to the active site of the cytochrome P-450scc.

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