Evidence for an ordered reaction mechanism for bile salt:3'phosphoadenosine-5'-phosphosulfate: sulfotransferase from rhesus monkey liver that catalyzes the sulfation of the hepatotoxin glycolithocholate[1]

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Abstract The in vivo formation of the sulfate ester of glycolithocholate is a critical step in the elimination of this hepatotoxic bile salt. Rhesus monkeys fed chenodeoxycholate or ursodeoxycholate, the precursors of lithocholate, develop frank cirrhosis in association with accumulation of nonsulfated glycolithocholate in bile. An enzyme catalyzing the formation of glycolithocholate-3-sulfate has been isolated from hepatic cytosol of adult female rhesus monkeys and has been purified 146-fold. When reduced it appears as a 30 kD band on an SDS-polyacrylamide gradient gel. It has a pH optimum of 7.0 and is stimulated by low concentrations of Mg^{2+} (up to 2 mM), but does not have an absolute requirement for this metal ion. The kinetics of this enzyme have been investigated to ascertain whether its reaction mechanism can account for the poor in vivo rate of glycolithocholate sulfation. Inhibitor studies with an oxidized metabolite of lithocholate, 3-keto-5β-cholanoate, showed that the latter is a competitive inhibitor of glycolithocholate and is noncompetitive with the active form of sulfate, 3'phosphoadenosine-5'-phosphosulfate. The monophosphonucleotide 3'-AMP is a competitive inhibitor of 3'phosphoadenosine-5'-phosphosulfate, and is noncompetitive with glycolithocholate. These observations are consistent with a sequentially ordered Bi Bi reaction mechanism in which the bile salt is the first substrate to bind to the enzyme. Such a reaction mechanism for bile salt:3'phosphoadenosine-5'-phosphosulfate:sulfotransferase would be, therefore, the first time in which the sulfate acceptor (the bile salt) is the initial substrate to bind to a sulfotransferase. These studies have shown that although rhesus monkeys have a liver enzyme capable of forming the sulfate ester of glycolithocholate, its reaction mechanism and the potent inhibition caused by simple metabolites, such as 3-keto-5β-cholanoate, may serve to underexpress the activity of the enzyme in vivo. —**Barnes, S., R. Waldrop, J. Crenshaw, R. J. King, and K. B. Taylor.** Evidence for an ordered reaction mechanism for bile salt:3'phosphoadenosine-5'-phosphosulfate:sulfotransferase from rhesus monkey liver that catalyzes the sulfation of the hepatotoxin glycolithocholate. *J. Lipid Res.* 1986. **27:** 1111–1123.

Supplementary key words bile salt • lithocholate • sulfotransferase enzyme assay • ion exchange • chromatofocusing • affinity chromatography • sequential mechanism • Bi Bi ordered mechanism • dead-end inhibitors.

The bile salt lithocholate, 3α-monohydroxy-5β-cholan-24-oate, is a product of anerobic bacterial metabolism of chenodeoxycholate, 3α,7α-dihydroxy-5β-cholan-24-oate, and its 7β-epimer, ursodeoxycholate in the large bowel (1). It is the major bile salt found in feces in humans (2). Both chenodeoxycholate and ursodeoxycholate have been used in humans for the pharmacological dissolution of cholesterol gallstones (3, 4). However, there is concern about the long-term use of these compounds because of the potentially harmful effects of lithocholate (5).

When lithocholate is administered in small doses to many species it causes hepatotoxicity, with portal inflammation and bile duct proliferation, reminiscent of histological lesions observed in human liver disease (6–10). In studies of possible hepatotoxicity arising from chronic administration of chenodeoxycholate, the rhesus monkey was used as the model of the human response. The finding of hepatic cirrhosis after 1 to 6 months of chenodeoxycholate administration (11) and of fetal toxicity in pregnant animals (12) initially caused great concern. However, clinical parallels in man were not observed. Subsequently,

Abbreviations: PAPS, 3'phosphoadenosine-5'-phosphosulfate; APS, adenosine-5'-phosphosulfate; 2,5-PAP, 2',5'-diphosphoadenosine; 3,5-PAP, 3',5'-diphosphoadenosine; 2'-AMP, 2'-adenosine monophosphate; 3'-AMP, 3'-adenosine monophosphate; 5'-AMP, 5'-adenosine monophosphate. Lithocholate, chenodeoxycholate, ursodeoxycholate, deoxycholate, cholate: trivial names for 3α-monohydroxy-5β-cholan-24-oate, 3α,7α-dihydroxy-5β-cholan-24-oate, 3α,7β-dihydroxy-5β-cholan-24-oate, 3α,12α-dihydroxy-5β-cholan-24-oate, and 3α,7α,12α-trihydroxy-5β-cholan-24-oate, respectively. Bile salt sulfotransferase: the name given to the class of bile salt: 3'phosphoadenosine-5'-phosphosulfate:sulfotransferases, one of which, taurolithocholate sulfotransferase (E.C. 2.8.2.14) has been purified from rat liver (19). TLC, thin-layer chromatography.

[1] Some of the data in this manuscript was presented at the American Association for the Study of Liver Diseases Annual Meeting in Chicago, IL in November 1984 and has appeared in abstract form (*Hepatology.* 1984. **5:** 1065).

analysis of the biliary bile salts in control and chenodeoxycholate-treated rhesus monkeys revealed that there was a marked accumulation of lithocholate (15% of total biliary bile salts) in treated animals (13, 14).

In humans, lithocholate does not undergo an efficient enterohepatic circulation (15), and therefore does not accumulate in the way that deoxycholate, 3α,12α-dihydroxy-5β-cholan-24-oate, the bacterial product of cholate, 3α,7α,12α-trihydroxy-5β-cholan-24-oate, does. The difference between lithocholate and other bile salts is attributed to the extensive, enzymatically catalyzed formation of its 3α-sulfate ester (16), a metabolite that has two sites of negative charge and which cannot be actively transported by the ileal bile salt active transport system (17). Sulfation, therefore, prevents lithocholate from undergoing an efficient enterohepatic circulation.

Hepatic bile salt:3′phosphoadenosine-5′-phosphosulfate: sulfotransferase activity has been previously demonstrated in several species, man (18), rats (19, 20), hamsters (21, 22), guinea pigs (23), and, to a limited extent, rabbits (S. Barnes, R. Waldrop, and J. G. Spenney, unpublished observations). The failure of rhesus monkeys to prevent accumulation of lithocholate and its conjugates when they are treated with chenodeoxycholic acid prompted us to investigate whether bile salt sulfotransferase was present in rhesus monkey liver and, if so, to characterize its chemical and enzymatic properties and its reaction mechanism.

Reaction mechanisms of sulfotransferases have not been systematically studied. The complete reaction mechanism and kinetics have been described in only a few cases (24–29). Recently, Chen and Segel (29) have reported that human liver bile salt sulfotransferase has a sequential random bi bi reaction mechanism. They used product inhibition and a dead-end inhibitor to reach this conclusion. Banerjee and Roy (25) concluded from substrate and product inhibition studies that guinea pig liver phenol sulfotransferase has a rapid equilibrium, random Bi Bi reaction mechanism. Pennings, Vrielink, and Van Kempen (26), using substrate product inhibition and dead-end inhibitors, have shown that the reaction mechanism for rat brain phenol sulfotransferase is sequential ordered Bi Bi, with binding of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to the enzyme first, followed by the sulfate acceptor (26). Duffel and Jakoby (27) used substrate and product inhibition and a dead-end inhibitor to show that phenol sulfotransferase from male rat liver has a rapid equilibrium, random Bi Bi mechanism with two dead-end product inhibitor complexes. Anhalt et al. (28) studied cat and rabbit liver sulfotransferases and could not decide, on the basis of substrate and product inhibition experiments only, whether the sequential mechanism involved was random or ordered.

The studies described herein have shown that the intrinsic activity of this enzyme is present in amounts comparable to that found in human liver and has similar enzymatic properties. Therefore, lack of bile salt sulfotransferase is not an explanation for the marked differences in lithocholate metabolism in humans and rhesus monkeys. The reaction scheme, a sequential order Bi Bi mechanism, in which the bile salt substrate binds first, has not been previously described for other sulfotransferases. Moreover, the potent inhibitory properties of 3-keto-5β-cholanoate, an oxidation product of lithocholate, may account for the poor rate of sulfation of lithocholate in vivo.

METHODS AND MATERIALS

Materials

Glycolithocholic acid, 2′,5′-diphosphoadenosine (2,5-PAP), 3′5′-diphosphoadenosine (3,5-PAP), 2′-, 3′-, and 5′-adenosine monophosphates, ADP, ATP, and adenosine-5′-phosphosulfate (APS) were obtained from Sigma Chemical Co., St. Louis, MO. Other bile acids were purchased from Steraloids, Wilton, NH. [11,12-^{3}H]Lithocholate (2 Ci/mmol) was purchased from Amersham-Searle, Arlington Heights, IL. Unlabeled 3′phosphoadenosine-5′-phosphosulfate (PAPS) was obtained from P-L Biochemicals, Milwaukee, WI, and ^{35}S-labeled PAPS (1 Ci/mmol) was from New England Nuclear Corp., Boston, MA. The latter was freeze-dried to remove ethanol added by the manufacturer and redissolved in water. Its radiopurity was checked regularly by electrophoresis on paper strips in a pyridine-acetic acid buffer, pH 5.3 (20). Dicyclohexyl carbodiimide, ethyl chloroformate, and tri-N-butylamine were obtained from Aldrich Chemical Co., Milwaukee, WI. Solvents were the best grades available and were distilled before use. Butan-1-ol was purchased from Fisher Chemical Co., Norcross, GA. Aquasol liquid scintillation fluid was bought from Amersham-Searle Corp., Arlington Heights, IL. Sephacryl S-200 and the chromatofocusing materials, PBE-94 and polybuffer 74, were purchased from Pharmacia Fine Chemicals Ltd., Piscataway, NJ. DEAE-cellulose (DE-52) was obtained from Whatman, Clifton, NJ. 3′,5′-Diphosphoadenosine-hexane-agarose (PAP-agarose) affinity phase was purchased from P-L Biochemicals.

Synthesis of bile salt derivatives

Glyco-[11,12-^{3}H]lithocholate was synthesized from [11,12-^{3}H]lithocholate by the mixed anhydride procedure of Norman (30). After mild alkaline hydrolysis of the ethyl ester, the acid form was extracted in diethyl ether at pH 6. It was purified by TLC on 250-μm thick layers of silica gel G coated on 20 × 20 cm glass plates (Fisher Chemical Co., Norcross, GA) with the developing solvent, hexane-

ethyl acetate–acetic acid–propan-2-ol 20:10:10:1 (by vol). Its purity was checked with two other solvent systems, 1,2-dichloroethane–acetic acid–water 10:10:1 (by vol) and chloroform–methanol–3 M ammonium hydroxide 60:30:4 (by vol). Bile salt sulfate esters were synthesized by dicyclohexylcarbodiimide procedure of Mumma (31), as described elsewhere (20, 21).

Animals

Rhesus monkey livers were obtained from several sources. Frozen livers from two male and one female rhesus monkeys were purchased from PelFreez (Rogers, AR). Livers from two anesthetized female monkeys were excised just prior to systemic perfusion of the brain with radioactive microspheres. Two female (5 kg) rhesus monkeys, previously used for studies of gonadotrophin secretion during the estrus cycle, and two female animals, retired from the breeding program at Litton Bionetics, were used for pharmacokinetic studies (S. Barnes, R. Waldrop, J. Crenshaw, and L. S. Frawley, unpublished data). At the end of these experiments they were killed and their livers were excised to be used in these studies.

Tissue preparation

Livers were homogenized (2 ml/g) in ice-cold 10 mM sodium phosphate–5 mM magnesium sulfate buffer–250 mM sucrose, pH 7.0 (buffer A). Differential centrifugation (21) was used to obtain soluble and particulate fractions of the homogenates. However, sulfotransferase activity was confined to the soluble supernatant fraction. In most cases, the supernatant was used immediately. However, storage at −20°C did not affect enzyme activity or its properties.

Chromatography procedures

Prior to ion-exchange chromatography on DEAE-cellulose, the supernatant fraction was diluted with distilled water until it had the same conductance as 10 mM sodium phosphate–5 mM magnesium sulfate buffer, pH 7.0 (buffer B). The diluted supernatant was mixed with 20 g DEAE-cellulose for 30 min at 4°C. Nonabsorbed proteins were removed by filtration and the pellet was further eluted with buffer B (3 × 100 ml). Bile salt sulfotransferase activity, which was completely adsorbed, was eluted with 2 × 50 ml of buffer B containing 200 mM NaCl. The combined eluates were dialyzed overnight against buffer B and then centrifuged at 105,000 *g* for 60 min at 4°C. The supernatant was loaded onto 10 g DEAE-cellulose equilibrated with buffer B column at 30 ml/hr. Nonabsorbed proteins were washed off with 100 ml of buffer B. The bile salt sulfotransferase activity was eluted by a 250-ml 0–200 mM NaCl gradient in buffer B. Elution of the column with up to 2 M NaCl produced no further bile salt sulfotransferase activity. The protein concentration in the eluted fractions was determined by the absorbance at 280 nm. NaCl concentration was determined by measurement of conductivity.

Chromatofocusing was carried out using a 15 × 1 cm PBE-94 column, equilibrated with 25 mM imidazole–HCl buffer, pH 7.4 (buffer C). Supernatant fractions (1 ml) were dialyzed against buffer C and centrifuged to remove fines. After loading the sample, nonbound proteins were eluted with three column volumes of buffer C. Bile salt sulfotransferase activity was eluted on a pH gradient from pH 7.4 to pH 3.7 by passing 155 ml of diluted polybuffer 74, pH 3.5 (15.5 ml of neat polybuffer 74 and 139.5 ml of degassed distilled water) at 10 ml/hr through the column.

Gel filtration was performed on a 90 × 1.5 cm Sephacryl S-200 column, as previously described (20).

Affinity chromatography was performed on 1 g of 3,5-PAP-hexane–agarose columns equilibrated with buffer B. Samples (cell supernatant and other fractions) to be loaded were dialyzed against buffer B. After washing nonabsorbed proteins from the column, absorbed proteins were eluted with buffer B containing 500 mM NaCl or 100 μM PAPS.

SDS-Polyacrylamide gel electrophoresis was carried out as described by Laemmli (32) using a 0.5-mm-thick 5–20% (w/v) linear gradient gel, made in 0.4 M Tris–HCl buffer, pH 8.8, containing 0.1% (w/v) SDS. Samples were denatured at 100°C for 3 min in 0.1 M Tris–HCl buffer, pH 6.8, containing 2% SDS, 0.28 M 2-mercaptoethanol, 12% (w/v) sucrose, and 0.002% (w/v) bromophenol blue. Aliquots (20 μl) were placed in the wells of a 3% (w/v) polyacrylamide stacking gel formed on top of the analytical gel. The samples were electrophoresed at a constant current of 25 mA at 4°C for 3 hr. After fixing the gel in 50% methanol–10% acetic acid, the proteins were located using the silver stain method of Merrill et al. (33).

Assay of sulfotransferase activity

Two radiotracer assay procedures were used. In competition experiments between glycolithocholate and other bile salts, glyco-[11,12-^{3}H]lithocholate was used in a manner similar to that described elsewhere (20, 21), except that the reaction was terminated by boiling, rather than by methanol-induced protein precipitation. Ammonium hydroxide (20 μl) was added after cooling and the precipitated protein was removed by centrifugation (3000 *g* for 10 min). Twenty μl of the supernatant was spotted onto a 20 × 20 cm silica gel G-coated glass TLC plate and developed with the solvent system, chloroform–methanol–acetic acid–water 65:24:15:6 (by volume). Zones corresponding to glycolithocholate-3-sulfate and unreacted glycolithocholate were scraped into scintillation vials for determination of radioactivity.

Most of the kinetic studies of sulfotransferase activity were carried out using ^{35}S-labeled PAPS, in which the

transfer of ^{35}S-sulfate to the substrate was measured. Separation of [^{35}S]PAPS and the ^{35}S-labeled product was carried out by an alkaline butanol extraction procedure which we have recently described (34). The details of the kinetic experiments are presented in the Kinetic Experiments section and in the Results section. All experiments were performed in 100 mM sodium phosphate buffer, pH 7.0, containing 2.5 mM magnesium sulfate. Glycolithocholate solutions were prepared in methanol containing 1% (v/v) 8 M ammonium hydroxide. Appropriate aliquots were transferred to 10 × 75 mm glass test tubes and the methanol and ammonia were removed by a stream of air. The buffer solution and the PAPS (0.1 μCi) were added, along with the nucleotide inhibitor (PAP, APS, etc.). The tube and contents were placed in an ultrasonication bath to effect solution of all components. The enzyme was added, usually as an equal volume, to bring the total volume up to 100 μl. The reaction was stopped by placing the tubes in a boiling water bath for 2 min. Sulfated products were extracted into butanol at pH 10 which was backwashed with 100 mM sodium phosphate, pH 7–1 M ammonium hydroxide 1:4 (v/v). Radioactivity in 0.8 ml of the butanol phase was determined by scintillation counting as described elsewhere (34).

Assay optimization

Optimum pH for each substrate was determined in 100 mM sodium phosphate buffers, ranging from pH 5.0 to pH 8.0. Incubation time was 60 min at 37°C in the presence of 5 mM $MgSO_4$. Optimum Mg^{2+} concentration was determined under similar incubation conditions at the optimum pH. Tested fractions were extensively dialyzed against 100 mM sodium phosphate buffer, pH 7.0. In one of the test incubations 1 mM EDTA was added. The incubation time over which the reaction rate was linear was tested using 100 μg of supernatant protein. The range of added supernatant protein over which the sulfotransferase activity increased linearly was determined using the optimized time. In all assays, including the kinetic experiments, the incubation time was adjusted so that less than 10% of the substrates were consumed.

Kinetic experiments

The experiments were designed so that the results could be analyzed in terms of the methods described by Cleland (35). The concentration of glycolithocholate was varied from 1 to 20 μM, and that of PAPS from 1 to 20 μM. Various potential inhibitors, 2,5-PAP, 3,5-PAP, 2-AMP, 3-AMP and 5-AMP, ADP, ATP and APS and numerous bile acids, were tested for inhibitor properties in an assay with fixed concentration of glycolithocholate (20 μM) or PAPS (22.5 μM) and various amounts of the other substrates. The phosphonucleotides were not sulfated under the assay conditions used; however, bile salts that were inhibitors and were themselves sulfated were also extracted into butanol, thus rendering them inappropriate for these studies. Only those bile salts that were not sulfated on their own, but which inhibited the sulfation of glycolithocholate, were used for further studies.

Treatment of kinetic data

The initial enzyme velocity was determined for each set of conditions. The data for the effect of varying the concentration of each substrate (A is glycolithocholate and B is PAPS) were fitted using the nonlinear program SEQUEN (35) to the equation:

$$v = V/\{K_{mA}/[A] + K_{mB}/[B]\,(1 + K_{iA}/[A]) + 1\} \qquad Eq.\ 1)$$

to obtain estimates of K_{mA} (the K_m of glycolithocholate), K_{mB} (the K_m of PAPS) and K_{iA} (the dissociation constant for the formation of the enzyme:glycolithocholate complex). Data from inhibition experiments were fitted to each of the following equations:

$$\text{Competitive} \quad v = VA/K_m\,\{(1 + I/K_{is}) + A\} \qquad Eq.\ 2)$$

$$\text{Uncompetitive} \quad v = VA/\{K_m + A(1 + I/K_{ii})\} \qquad Eq.\ 3)$$

$$\text{Noncompetitive} \quad v = VA/\{K_m(1 + I/K_{is}) + A(1 + I/K_{ii})\} \qquad Eq.\ 4)$$

where A is the substrate (glycolithocholate or PAPS) which is varied (the other substrate is held at constant concentration), I is the concentration of inhibitor, K_{ii} is the inhibition constant on the intercept, and K_{is} is the inhibition constant on the slope of a double reciprocal plot. All data fitting was done with the programs outlined by Cleland (35). A given model was rejected when one or more of the evaluated parameters was unreasonable, i.e., not significantly different from zero, when another model gave decidedly better standard errors for evaluated parameters, or when a simpler model did not result in a significant decrease in error sums of squares.

RESULTS

Distribution of bile salt sulfotransferase activity

Sulfotransferase activity, with each of the bile salts used as substrates, is confined to the cytoplasmic soluble fraction of livers and in female rhesus monkeys (43.0 ± 4.4 pmol · min^{-1} · mg protein^{-1}, mean ± SD, n = 5) is 40–50% of the values found in rats and in humans (36). The activity in male rhesus monkey liver is somewhat lower (11.0 ± 4.4 pmol · min^{-1} · mg protein^{-1}, mean ± SD, n = 3). However, the livers from the male animals were each purchased from a commercial supplier, whereas most of the livers from female animals were obtained fresh at this Institution. No bile salt sulfotransferase activity was detected in kidneys.

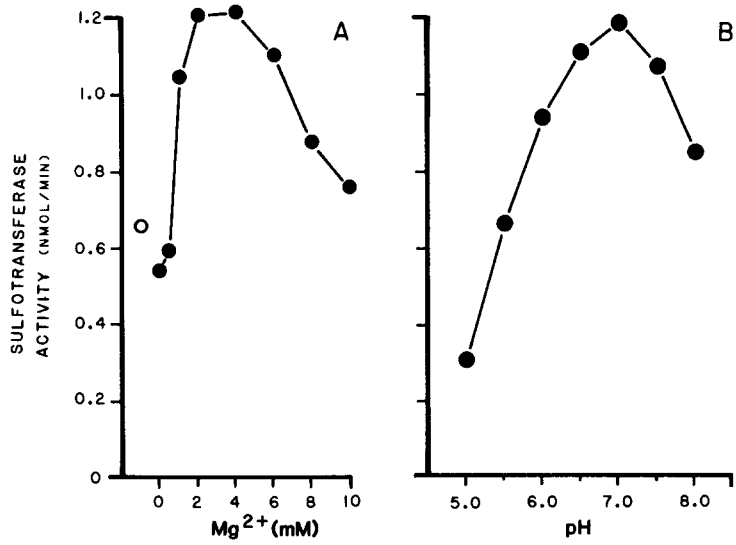

Fig. 1. A, Optimization of Mg^{2+} concentration. The Mg^{2+} concentration was varied between 0 and 10 mM. Incubations were carried out for 60 min at 37°C with 90 μM PAPS, 0.1 μCi of ^{35}S-labeled PAPS, 100 μM glycolithocholate, and 100 μg of supernatant protein. B, Optimization of pH for sulfotransferase activity. The pH was varied between 5.0 and 8.0 using 100 mM sodium phosphate buffers. Other incubation conditions were the same as in A except that the $MgSO_4$ concentration was 2.5 mM. The open circle represents the sulfotransferase activity in the presence of excess EDTA.

Assay conditions

Optimization of bile salt sulfotransferase activity showed that it is greatest at pH 7.0 for glycolithocholate (**Fig. 1A**).

Bile salt sulfotransferase activity is stimulated (122%) by the addition of Mg^{2+} up to 2.5 mM (Fig. 1B). EDTA does not diminish bile salt sulfotransferase activity in incubations without added Mg^{2+}; indeed, there is a small increase (20%). Higher concentrations of Mg^{2+} inhibit bile salt sulfotransferase activity.

The production of glycolithocholate-3-sulfate increases linearly for 60 min. Using a 60-min incubation period, the observed reaction rate increases with corresponding increase in the amount of added 105,000 *g* supernatant fraction, up to 100 μg of supernatant protein. These data are very similar to those we recently reported for rat liver bile salt and steroid sulfotransferases (34).

Partial purification

Bile salt sulfotransferase behaves as a single enzyme activity during chromatographic fractionation (**Table 1**). It is eluted as a single peak from a DEAE-cellulose column by a 0–200 mM NaCl gradient at 80 mM NaCl (**Fig. 2**). Gel filtration shows that it is a protein of apparent molecular weight 37,000 which increases to 65,000 in the presence of 5 mM 2-mercaptoethanol (**Fig. 3**). Chromatofocusing also produces a single peak of activity with an apparent pI of 5.25 (**Fig. 4**). Attempts to adsorb the bile salt sulfotransferase activity in the soluble supernatant fraction onto the 3,5-PAP-hexane–agarose affinity phase have been unsuccessful. However, when bile salt sulfotransferase, partially purified by DEAE-cellulose and chromatofocusing, is used, some adsorption does occur to the affinity phase. When the enzyme activity which does not bind to this affinity column is passed over a second PAP–hexane–agarose column, a similar proportion of the enzyme activity is bound to the column. The bound enzyme activity is eluted by 0.5 M NaCl and represents 146-fold purification over the activity in the soluble supernatant fraction. After dialysis to remove the NaCl, the fraction is used for kinetic studies. SDS-polyacrylamide gel electrophoresis of the affinity gel purified enzyme, eluted by PAPS (100 μM) and denatured by boiling in 1% SDS under reducing conditions, indicates that there were two major protein bands (30,000 and 53,000), the former being the bile acid sulfotransferase (**Fig. 5**).

Kinetic constants in uninhibited systems

The apparent K_m and V_{max} of both glycolithocholate and PAPS have been determined in a series of experiments

TABLE 1. Purification of rhesus monkey liver bile salt sulfotransferase

Step	BAST	Protein	Specific Activity	Purification
	nmol/hr	*mg*	*units/mg*	
Cell supernatant	603.3	1208	0.50	1
DEAE-cellulose	591.4	145	4.08	8.2
Chromatofocusing	727.4	67	10.87	21.8
Affinity column	38.6	0.53	72.8	146

Since less than 15% of the enzyme activity is initially adsorbed from the peak from the chromatofocusing step, recovery from the affinity column is approximately 40%.

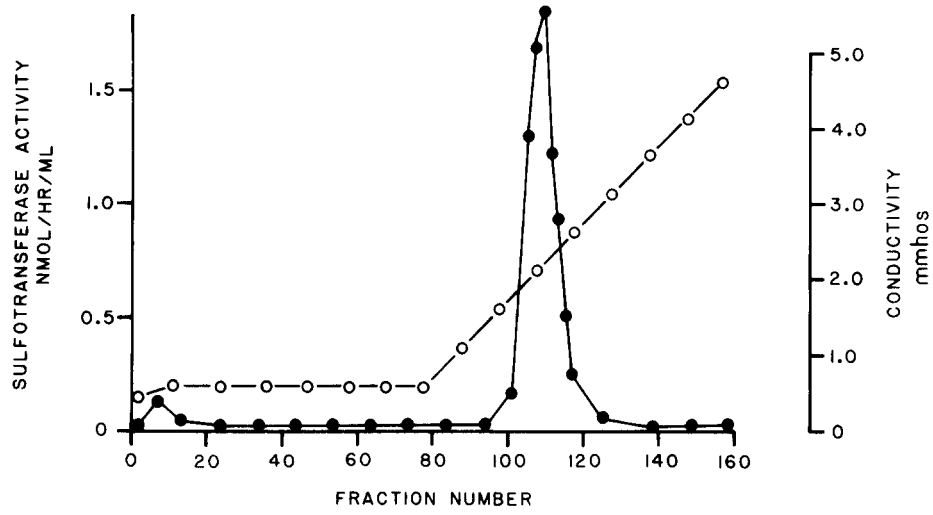

Fig. 2. DEAE-cellulose chromatography of rhesus monkey liver sulfotransferase activity. One ml of supernatant dialyzed against 10 mM sodium phosphate buffer, pH 7.0, was loaded onto a 20 × 1 cm DE-52 column equilibrated with the same buffer. Unbound proteins were eluted with this buffer. Bound proteins were eluted by passing a 200-ml gradient of 0–200 mM NaCl in the loading buffer over the column at 10 ml/hr; (● —— ●) sulfotransferase activity; (○ —— ○) conductance.

in which one substrate is the variable substrate and the other is the constant variable substrate (1–20 μM for PAPS and 1–20 μM for glycolithocholate). Determination of enzyme velocity as a function of the concentration of the other substrate reveals that the sulfotransferase reaction mechanism is sequential, as opposed to a ping-pong mechanism, since the double reciprocal plots intersect to the left of the abcissa (**Fig. 6**). In studies on the partially purified preparation of the enzyme, the K_m for PAPS is 9.87 ± 1.16 μM and the K_m for glycolithocholate is 6.67 ± 0.66 μM. The K_i for PAPS binding to the enzyme complex is 2.98 ± 0.43 μM and for glycolithocholate binding is 2.12 ± 0.28 μM.

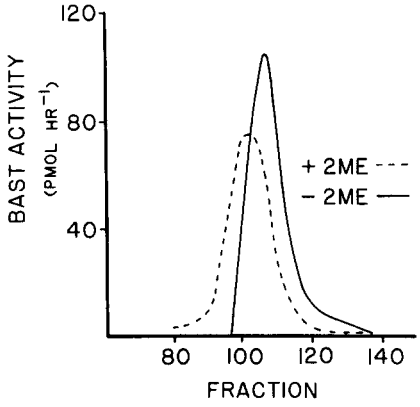

Fig. 3. Gel filtration of rhesus monkey liver sulfotransferase activity. Supernatant (0.5 ml) in 100 mM sodium phosphate–5 mM $MgSO_4$ buffer, pH 7.0, was applied to a 90 × 1.5 cm column of Sephacryl S-200 equilibrated with the same buffer. Sulfotransferase activity was eluted with this buffer at 0.33 ml/min (——). In a second experiment, 2 mM 2-mercaptoethanol was added to the supernatant prior to chromatography; elution was also carried out with the same concentration of 2-mercaptoethanol in the eluant (- - -).

Kinetics in inhibited reactions

Many bile salts have been studied for their potential as bile salt sulfotransferase inhibitors. It should be made clear that when using the alkaline butanol extraction procedure a bile salt inhibitor must itself not become sulfated since the assay would not distinguish between this bile salt and glycolithocholate. Of 25 bile salts that have been tested on a cytosol preparation of the enzyme, the most inhibitory is 3-keto-5β-cholanoate (**Table 2**). It inhibits 75% of bile salt sulfotransferase activity when incubated with equimolar amounts of glycolithocholate, and is not sulfated itself. The 5α- and 5β-forms of 3,6-diketo-5(H)-cholanoate are also inhibitors (51.6% and 29.3%, respectively) that are not sulfated.

Inhibition studies have been carried out using 3-keto-5β-cholanoate. In the first experiment, at a constant PAPS concentration (5 μM), the glycolithocholate concentration was varied from 1 to 20 μM in the presence of inhibitor (0–20 μM). The double reciprocal plot demonstrates that the data fit the competitive model (equation 2) (**Fig. 7**). Replots of $1/V_{max}$ and K_m/V_{max} versus the inhibitor concentration are constant for the former and increase linearly with inhibitor concentration for the latter (Fig. 7, inset). The K_i for 3-keto-5β-cholanoate is 0.55 ± 0.05 μM.

In the second experiment, at a constant glycolithocholate concentration (5 μM), the PAPS concentration was varied from 1 to 20 μM in the presence of inhibitor (0–20

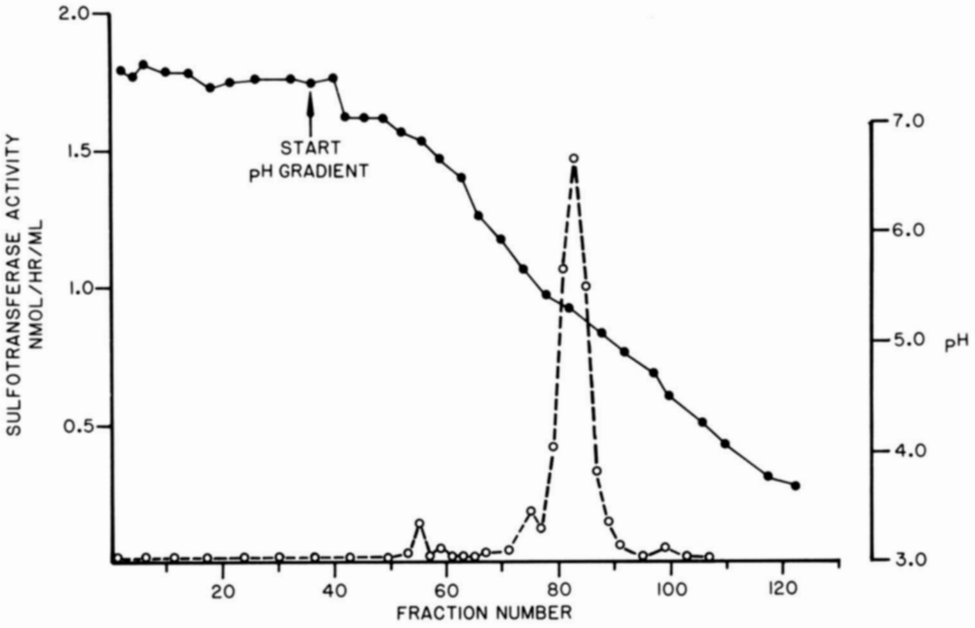

Fig. 4. Chromatofocusing of rhesus monkey liver sulfotransferase activity. One ml (100 mg of protein) of supernatant was dialyzed against 25 mM imidazole-HCl buffer, pH 7.4, and then applied to a 15 × 1 cm PBE 94 column, equilibrated with this buffer. Proteins with pI values above 7.0 were washed off the column with the starting buffer. Bound proteins were eluted on a linear pH gradient from 7.4 to 3.5 by passage of 155 ml of diluted polybuffer 74, pH 3.5 (see Methods for details); (● — ●) pH; (○ - - - ○) sulfotransferase activity.

μM). Analysis of these data reveals that 3-keto-5β-cholanoate is a noncompetitive inhibitor of PAPS (equation 4); each of the double reciprocal plots intersect to the left of the abcissa (**Fig. 8**). Replots of the intercepts and slopes both increase linearly with the concentration of the inhibitor (data not shown). The K_{is} is PAPS is 2.33 ± 0.29 μM and the K_{ii} for 3-keto-5β-cholanoate is 0.85 ± 0.07 μM.

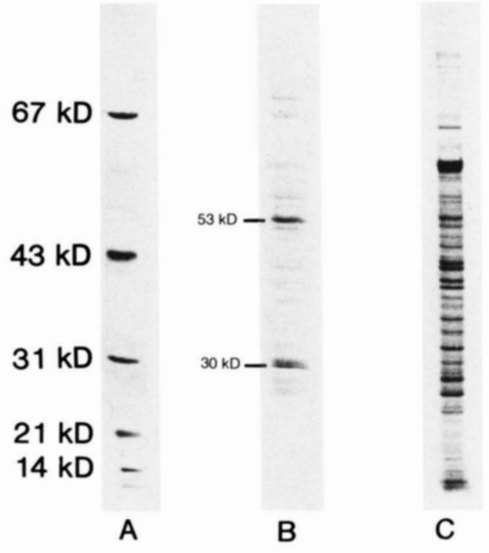

Fig. 5. SDS-Polyacrylamide gradient (5–20%) gel electrophoresis of denatured, reduced bile salt sulfotransferase from rhesus monkey liver. Proteins were visualized with a silver stain (33). Lane A contains molecular weight standards. Lane B contains the proteins that bound to the PAP-hexane-agarose affinity column and were eluted by PAPS; the 30 kD band is the bile salt sulfotransferase; the identity of the 53 kD band is unknown. Lane C contains the proteins which pass through the PAP-hexane-agarose affinity column (this includes some of the bile salt sulfotransferase activity).

A search was conducted to find a nucleotide inhibitor which competes with PAPS for binding to the substrate complex. These experiments have shown that 3,5-PAP, a product of sulfotransferase reactions, inhibits rhesus monkey liver bile salt sulfotransferase (**Table 3**). Interestingly, 2,5-diphosphoadenosine (2,5-PAP), a positional isomer of 3,5-PAP, is also an inhibitor (Table 3), albeit weaker than 3,5-PAP. However, both 2,5-PAP and 3,5-PAP produce complex inhibitory effects on PAPS and glycolithocholate kinetics and have not been studied further.

These data, along with the failure of ADP, ATP, and 5'-AMP to act as inhibitors, suggest that the 3'-position, and to lesser extent the 2'-position, rather than the 5'-position, is crucial for the binding of the phosphorylated nucleotides to the sulfotransferase. Accordingly, investigation of isomeric adenosine monophosphates has revealed that only 3'-AMP is an inhibitor (Table 2), although it is a less effective inhibitor than either 2,5-PAP or 3,5-PAP.

The effect of 3'-AMP (0–500 μM) on PAPS kinetics has been carried out in the presence of 5 μM glycolithocholate. Analysis of enzyme velocities has shown that 3'-AMP is a simple competitive inhibitor of PAPS (equation 2) (**Fig.**

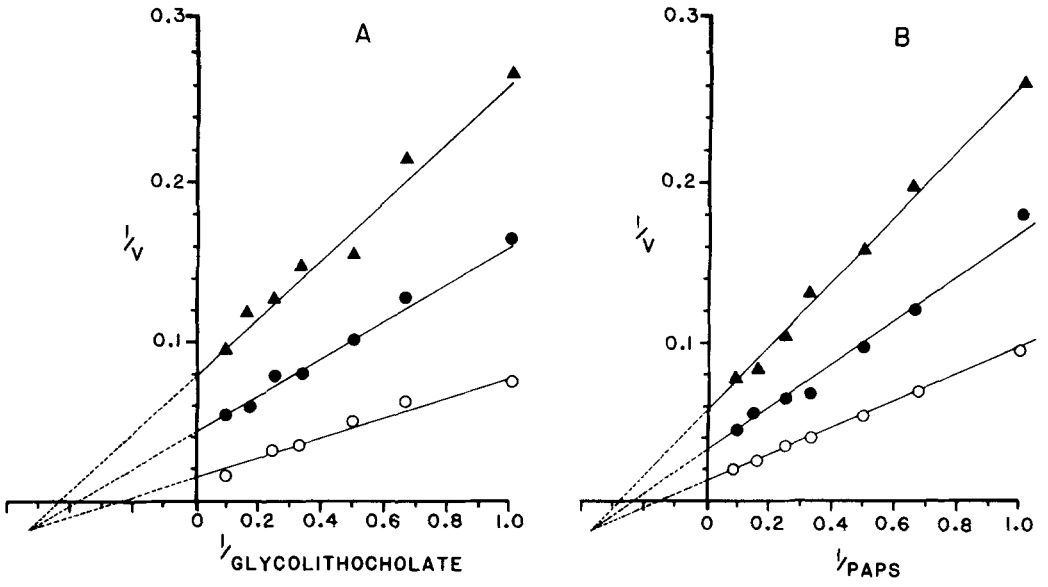

Fig. 6. Double reciprocal plots of 1/(sulfotransferase activity) versus 1/(substrate concentration). In A the PAPS concentration was constant, (▲) 1 μM, (●) 2.5 μM, or (○) 10 μM, in each study, and the glycolithocholate concentration was varied from 1 to 20 μM. In B the glycolithocholate was held constant, (▲) 1 μM, (●) 2.5 μM, or (○) 10 μM, in each study, and the PAPS concentration was varied from 1 to 20 μM.

9). Unlike 2,5-PAP and 3,5-PAP, it does not exhibit effects due to more than one binding site. The K_i for 3'-AMP is 455 ± 53 μM.

Finally, the effect of 3'-AMP on glycolithocholate kinetics has been examined. Analysis of enzyme velocities has revealed that 3'-AMP is an uncompetitive inhibitor of glycolithocholate (equation 3) (**Fig. 10**). Replots of slopes and intercepts versus inhibitor concentration have shown that slopes (K_m/V_{max}) are independent of the inhibitor concentration, whereas intercepts increase linearly (data not shown). The K_{ii} for 3'-AMP is 542 ± 39 μM.

A summary of the kinetic constants for substrates and inhibitors is given in **Table 4.**

TABLE 2. Bile salt inhibition of sulfation of glycolithocholate

Bile Salt	Relative Activity	% Inhibition
3-Keto-5β-cholanoate	0.0	74.6
3α-Hydroxy-6-keto-5α-cholanoate	1.77	42.5
3α-Hydroxy-6-keto-5β-cholanoate	0.49	41.7
3,6-Diketo-5α-cholanoate	0.03	51.6
3,6-Diketo-5β-cholanoate	0.0	29.3
3α,6α-Dihydroxy-5β-cholanoate	0.19	18.3
3α,6β-Dihydroxy-5β-cholanoate	0.02	19.3
3α,7α-Dihydroxy-5β-cholanoate	0.16	20.4
3α,7β-Dihydroxy-5β-cholanoate	0.54	16.4
3α-Hydroxy-7-keto-5β-cholanoate	0.19	17.6
3α,12α-Dihydroxy-5β-chol-8(14)-enoate	0.78	67.4
3α,12α-Dihydroxy-5β-cholanoate	0.63	63.0
3α-Hydroxy-12-keto-5β-cholanoate	0.78	77.3
3α-7α,12α-Trihydroxy-5β-cholanoate	0.01	0

The activity of rhesus monkey bile salt sulfotransferase was tested with the above bile salts, in the presence and absence of glycolithocholate. Each was added to give a final concentration of 100 μM. The PAPS concentration was 22.5 μM. One hundred μg of protein from the rhesus monkey liver soluble supernatant fraction was used as the source of sulfotransferase activity. Percentage inhibition is calculated from the observed and the expected sulfotransferase activity, the latter being based on the sum of the sulfotransferase activities with the test bile salt and glycolithocholate.

DISCUSSION

The hepatic cirrhosis that occurs in rhesus monkeys when they are treated with oral doses of chenodeoxycholate and ursodeoxycholate (1, 11), and the associated biliary accumulation of the bacterial metabolite lithocholate (14), was a disturbing observation. It aroused speculation that similar effects would be observed in human subjects with cholesterol gallstone disease who were being treated with chenodeoxycholate for gallstone dissolution. However, significant human liver toxicity has not been reported; most effects are mild in nature and are not sustained beyond 3 months of treatment (37).

Several reasons for the accumulation of lithocholate in the bile of rhesus monkeys could be considered. For instance, intestinal lithocholate production from chenodeoxycholate or ursodeoxycholate, due to the particular bacterial flora of the rhesus monkey, may be greater than in humans. Squirrel monkeys, which do not suffer from hepatoxicity following chenodeoxycholic acid administration (38), appear to have bacterial flora that convert chenodeoxycholate to lithocholate less efficiently than in man (39). Alternatively, lithocholate production could be

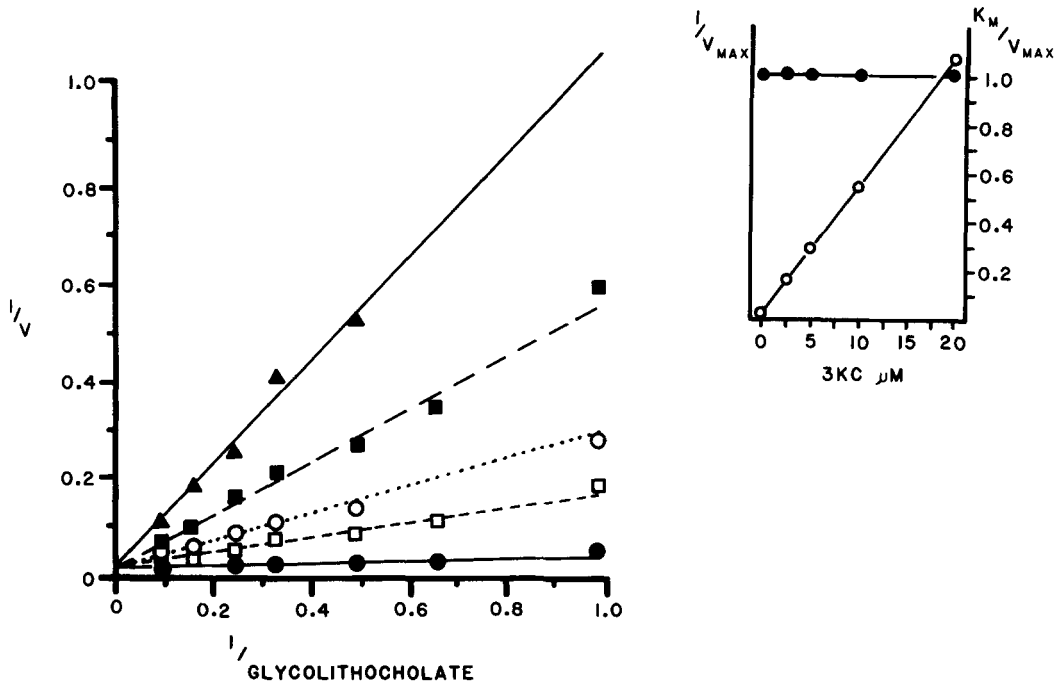

Fig. 7. Double reciprocal plots of 1/(sulfotransferase activity) versus 1/(glycolithocholate concentration) in the presence of various concentrations of 3-keto-5β-cholanoate; (●) 0 μM, (□) 2.5 μM, (○) 5 μM, (■) 10 μM, and (▲) 20 μM. The PAPS concentration was 5 μM in each study. Replots of intercepts ($1/V_{max}$) and slopes (K_m/V_{max}) against 3-keto-5β-cholanoate concentration are shown in the inset.

increased because the doses of chenodeoxycholate which cause hepatic damage (100 mg/kg per day) in rhesus monkeys are ten times higher than those used in humans.

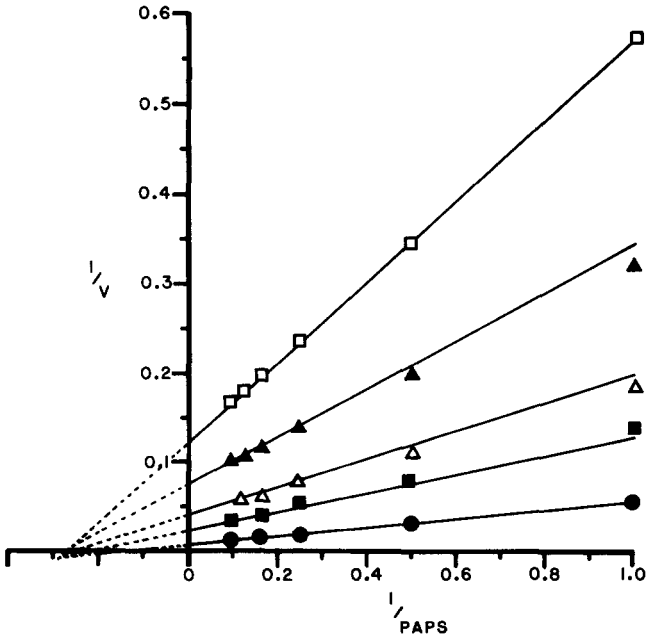

Fig. 8. Double reciprocal plots of 1/(sulfotransferase activity) versus 1/(PAPS concentration) in the presence of 3-keto-5β-cholanoate; (●) 0 μM, (■) 2.5 μM, (△) 5 μM, (▲) 10 μM, and (□) 20 μM. The glycolithocholate concentration was 5 μM in each study.

Despite each of these possibilities, when nonsaturating tracer doses of lithocholate or its glycine conjugate are administered intravenously, the conversion to the sulfate ester is 4–5 times lower in the rhesus monkey than in humans (40, 41). Therefore, lithocholate is metabolized more slowly at all doses. This may explain why untreated rhesus monkeys have evolved such a small pool of chenodeoxycholic acid; larger amounts would have been hepatotoxic. The crucial question is, therefore, why is lithocholate so poorly sulfated?

In this study, we have isolated a bile salt sulfotransferase from rhesus monkey liver that catalyzes the sulfation of glycolithocholate. It has properties similar to other bile salt sulfotransferases: its pH optimum is at 7.0 and it is stimulated by addition of Mg^{2+}, although Mg^{2+} is not an essential cofactor. Unlike rodent bile salt sulfotransferases (20, 42), it behaves as a single enzyme species when chromatographed on Sephacryl S-200, DEAE-cellulose, and a PBE-94 chromatofocusing column. SDS-polyacrylamide gradient gel electrophoresis of the denatured enzyme performed under reducing conditions indicates that it has a molecular weight of 30,000 similar to the bile salt sulfotransferase in rat liver (S. Barnes, L. Lagroue, and S. Buchina, unpublished observations). This value is a little lower than that obtained by Sephacryl S-200 gel filtration chromatography. The upward shift in apparent molecular weight following the addition of the reducing agent 2-mercaptoethanol using the latter technique is

TABLE 3. Inhibition of glycolithocholate sulfation by nucleotides

Nucleotide		Inhibition (%)[a]
2'-AMP		0
3'-AMP	(5)	12.5
5'-AMP	(6)	0
2',5'-PAP (50 μM)	(6)	37
2',5'-PAP (100 μM)	(6)	46
3,5'-PAP (40 μM)	(4)	54
APS		4
ADP		0
ATP		0

[a]Mean values.

The assay concentrations of glycolithocholate and PAPS were 30 μM and 22.5 μM, respectively. Each nucleotide concentration, except where noted, was 100 μM. Percentage inhibition was calculated using a control incubation with no added nucleotide except PAPS. The inhibition of each noninhibitory nucleotide was tested over a wide range (up to 1.5 mM) in duplicate. Additionally, certain nucleotides were studied in several separate experiments (number of experiments in parentheses).

similar to that observed for rat liver bile salt sulfotransferase (20), and may reflect dimerization following exposure of hydrophobic regions due to the breaking of intramolecular bridges. The detergent SDS presumably prevents hydrophobic interaction in the polyacrylamide gels, so that only the monomeric specie(s) is observed.

The mean intrinsic activity of rhesus monkey bile salt sulfotransferase measured in vitro is between a third and a half of that reported in human liver (36), although there is substantial overlap of individual values. Therefore, the lack of bile salt sulfotransferase in rhesus monkey liver is not an adequate explanation for the poor rate of lithocholate sulfation, since the overlap in enzyme activity ought to suggest that lithocholate toxicity would be encountered regularly in humans with liver disease.

The answer may lie in the kinetic properties of this enzyme, i.e., the reaction mechanism, affinity for its substrates, and the role of inhibitors which could be encountered in the in vivo situation.

In the present study we have shown that the sulfation of glycolithocholate occurs by a sequential ordered Bi Bi reaction mechanism (**Fig. 11**). It differs from that for other sulfotransferases with ordered mechanisms (25) in that the bile salt binds first to the enzyme, not the PAPS.

Several lines of evidence for the proposed reaction mechanism have been obtained. Firstly, the intersecting double reciprocal plots (Fig. 6) for the enzyme activity at various concentrations of one substrate in the presence of fixed concentrations of the other substrate have ruled out the possibility of a ping-pong mechanism and are consistent with a sequential mechanism (43). However, these data do not discriminate between an ordered or a random mechanism.

It is, therefore, necessary to use product and dead-end inhibitor studies to distinguish between the possible mechanisms. The bile salt 3-keto-5β-cholanoate is a competitive inhibitor of glycolithocholate and is a noncompetitive inhibitor of PAPS. The latter finding precludes the possibility of PAPS being the first substrate to bind to the enzyme, but does not allow distinction between a random mechanism or one in which glycolithocholate is the obligatory first substrate.

The poor binding of rhesus monkey bile salt sulfotransferase to the affinity matrix, 3,5-PAP-hexane-agarose, argues against the random mechanism. In contrast, this affinity matrix binds rat liver bile salt sulfotransferase very efficiently (S. Barnes, R. J. King, and M. Whitman, unpublished observations). The reaction product, 3,5-PAP, causes complex effects on the kinetics of PAPS and glycolithocholate. In contrast, 3,5-PAP is a competitive inhibitor of PAPS for rat liver hydroxysteroid sulfotransferase (44). This suggests that 3,5-PAP interacts with the rhesus monkey bile sulfotransferase in at least two steps in the reaction, possibly by forming an enzyme-bile salt-3,5-PAP dead-end complex, and by inhibiting the dissociation of the enzyme-bile salt sulfate-3,5-PAP product complex. This situation also demands that enzyme-3,5-PAP complexes do not form, and in turn shows that 3,5-PAP may be the first product to dissociate from the enzyme-products complex. The dissociation of the enzyme-products complex may, therefore, also be ordered.

Therefore, another nucleotide inhibitor was looked for that only had a simple, i.e., one site, inhibitory effect on PAPS kinetics. Nucleotides with substitution only in the

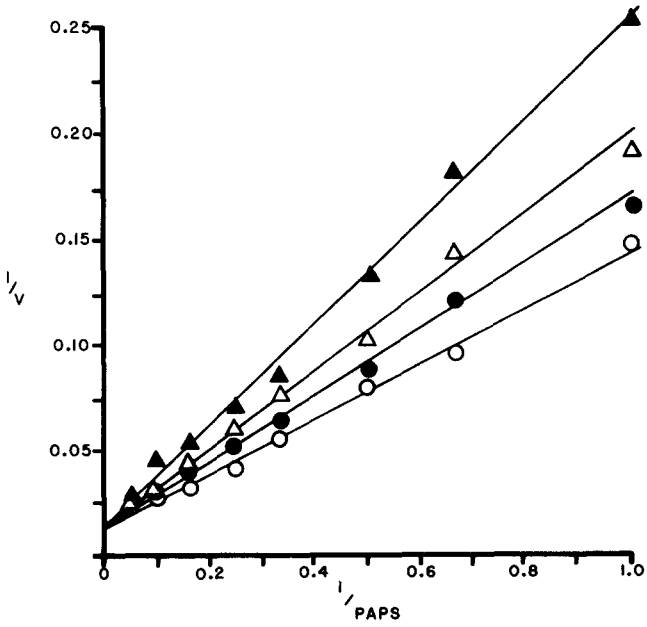

Fig. 9. Double reciprocal plot of 1/(sulfotransferase activity) versus 1/(PAPS concentration) in presence of 3'-AMP; (○) 0 μM, (●) 100 μM, (△) 200 μM, and (▲) 500 μM. The glycolithocholate concentration was 5 μM in each study.

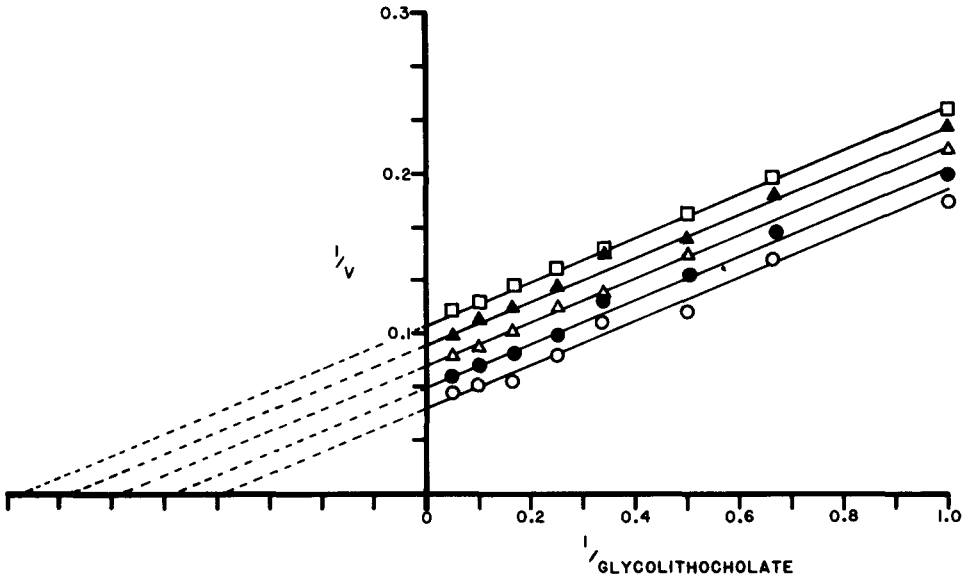

Fig. 10. Double reciprocal plot of 1/(sulfotransferase activity) versus 1/(glycolithocholate concentration) in the presence of 3'-AMP; (○) 0 μM, (●) 200 μM, (△) 400 μM, (▲) 600 μM, and (□) 800 μM. The PAPS concentration was 5 μM in each study.

5'-position (APS, ATP, and ADP) are not inhibitors of bile salt sulfotransferase, unlike bovine adrenal estrogen sulfotransferase (24), rat brain phenol sulfotransferase (26), and rat liver glucocorticoid sulfotransferase I (45). This suggests that the 3'-position is crucial for binding to the active site of the enzyme, as previously noted by Adams, Ellyard, and Low (24). Examination of the effects of the isomeric adenosine monophosphates have confirmed this, since only 3'-AMP has inhibitory properties. More importantly, 3'-AMP is a simple competitive inhibitor of PAPS (Fig. 9), which suggests that 3'-AMP binds to the enzyme–substrate complex rather than the product complex. Similar competitive effects of 3'-AMP on PAPS kinetics have been observed for bovine adrenal estrogen sulfotransferase (24).

The effect of 3'-AMP on glycolithocholate kinetics is crucial for the elucidation of the reaction mechanism. A random mechanism requires that noncompetitive inhibition should have occurred. However, 3'-AMP causes uncompetitive inhibition, consistent with obligatory initial binding of glycolithocholate to the enzyme (43).

Such a mechanism provides a rationale for the lack of inhibition of bile salt sulfotransferase activity by very high concentrations of glycolithocholate, since dead-end enzyme–3,5-PAP complexes do not form (as confirmed by poor binding to the PAP-affinity matrix). 3,5-PAP may bind to the enzyme–glycolithocholate complex, but has to do so in the face of a much higher (at least 10-fold) competing PAPS concentration, thereby minimizing any possible inhibition. Of the other sulfotransferases that have been studied, some exhibit substrate (phenol and steroid sulfotransferase) inhibition (26, 28, 46, 47), whereas other steroid sulfotransferases (45, 48) do not. It can be speculated that the latter sulfotransferases may have reaction mechanisms similar to that for rhesus monkey bile salt sulfotransferase.

The formation of dead-end complexes between bile salt inhibitors and the enzyme may have an important physiological role in rhesus monkeys, particularly since the most potent inhibitor, 3-keto-5β-cholanoate, is a possible metabolite of lithocholate itself. Since the K_i for 3-keto-5β-cholanoate was somewhat lower than the apparent K_m and K_i for lithocholate, significant inhibition of bile salt sulfotransferase activity is a serious possibility. In addition, bile salts with oxygen substituents at positions 6 and 7 are moderately good inhibitors, including both chenodeoxycholate and ursodeoxycholate, precursors of lithocholate. 3-Keto bile salts, formed by intestinal bacteria, are found in portal blood in humans (49). Rhesus

TABLE 4. Kinetic parameters for rhesus monkey liver bile salt:PAPS:sulfotransferase

Bile Salt	Constant	Mean ± SEM, units μM
Glycolithocholate	K_m	6.67 ± 0.66
Glycolithocholate	K_i	2.12 ± 0.28
PAPS	K_m	9.87 ± 1.16
PAPS	K_i	2.98 ± 0.43
PAPS	K_{is}	2.33 ± 0.29
3-Keto-5β-cholanoate	K_{is}	0.55 ± 0.05
3-Keto-5β-cholanoate	K_{ii}	0.85 ± 0.07
3-AMP	K_{is}	455 ± 53
3-AMP	K_{ii}	542 ± 39

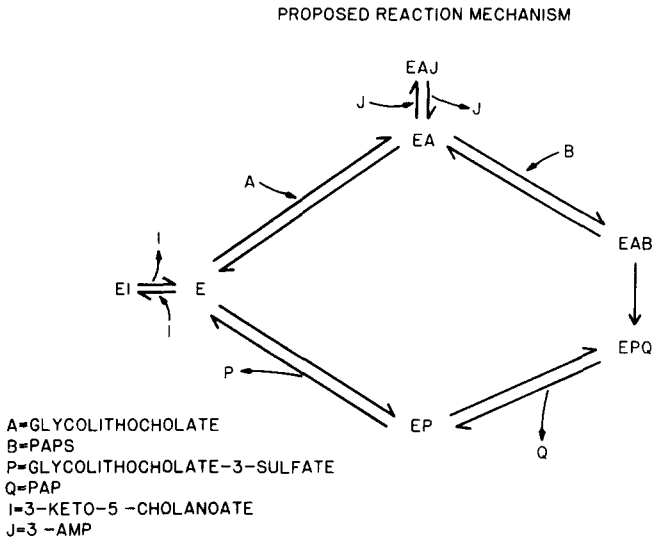

Fig. 11. Proposed reaction mechanism for rhesus monkey liver glycolithocholate (bile salt):PAPS:sulfotransferase.

monkey liver contains a 3α-hydroxysteroid dehydrogenase (50) which should convert 3-keto bile salts to their 3-hydroxy forms. However, it is not yet known whether its activity in vivo matches that measured in in vitro experiments.

In those animals in which alternative metabolic pathways for lithocholate exist, inhibition of lithocholate sulfation would not lead to hepatotoxicity. This is particularly true of the rat which can tolerate large doses of lithocholate by carrying out 6β-hydroxylation of lithocholate (9). Pigs, which have no hepatic bile salt sulfotransferase activity (S. Barnes and R. Waldrop, unpublished observations), convert lithocholate to 3α,6α-dihydroxy-5β-cholanoate (hyodeoxycholate) (51). On the other hand, baboons, which convert lithocholate to its sulfate ester less well than rhesus monkeys (52) and suffer from chenodeoxycholate-induced hepatoxicity, must lack such alternative metabolic pathways. All these studies highlight the problems faced in trying to find an adequate animal model to evaluate the metabolism and toxicity of bile salts.

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