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ENZYMATIC SULFATION OF BILE SALTS

PARTIAL PURIFICATION AND CHARACTERIZATION OF AN ENZYME FROM RAT LIVER THAT CATALYZES THE SULFATION OF BILE SALTS

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Summary

An enzyme system which catalyzes the transfer of sulfate group from 3′-phosphoadenosine-5′-phosphosulfate to bile salts has been identified and characterized from rat liver. The enzyme is present in the cytosol fraction of liver cells. The apparent K_m value for 3′-phosphoadenosine-5′-phosphosulfate was $8 \cdot 10^{-6}$ M and that for taurolithocholate was $5 \cdot 10^{-5}$ M. Sulfation occurred with conjugated as well as unconjugated bile salts, however the rate of sulfation was higher with conjugated than unconjugated. The enzyme was present in rat liver and kidney, but not detectable in brain, lung, heart, spleen or intestinal mucosa. The activity is completely inhibited by *p*-chloromercuribenzoate indicating the enzyme requires a sulfhydryl group for activity. A molecular weight of 130 000 was estimated by gel-filtration technique and the enzyme shows an isoelectric point of 5.3.

Until recently bile salts were thought to be metabolic end-products. The first indication that these compounds underwent further structural modification was provided by Palmer [1]. He observed that a significant amount of ^{14}C-labelled lithocholate in the bile was converted to a more polar metabolite in patients with cholelithiasis. Subsequent work has shown that this metabolite is the 3α-sulfate ester of lithocholate and that 40—70% of lithocholate in the bile is sulfated [2,3].

Studies of subjects with intrahepatic cholestasis and individuals with alcoholic liver disease showed that sulfation might be important in the metabolism of bile salts [4]. Extensive formation and renal excretion of the sulfate esters

of the four major bile salts (lithocholate, deoxycholate, chenodeoxycholate and cholate) occurred in these patients. Moreover, the apparent renal clearances of bile salt sulfates were several hundred times greater than the clearances of non-sulfated bile salts. Normal subjects also have small quantities of bile salt sulfates present in their urine and peripheral blood [5]. Thus, these investigations have established in human that bile salts are metabolized to sulfate esters.

Furthermore, studies with animals also demonstrate the possible importance of sulfation in the metabolism of bile salts. One such study showed that the metabolism of taurolithocholate sulfate differs significantly from non-sulfated forms. The fractional intestinal absorption of taurolithocholate sulfate is less than the fractional absorption of non-sulfated forms [6]. In addition, renal excretion of glyco- or taurolithocholate sulfate is greater than excretion of non-sulfated glyco- or taurolithocholate [4].

By increasing urinary and fecal excretion of bile salts, sulfation may possibly retard the accumulation of potentially toxic concentrations of these compounds. For example, lithocholate has been shown to be extremely hepatotoxic in animals [7] and may be important in the pathogenesis of human disease. The formation and extensive excretion of lithocholate sulfate may be a normal mechanism to protect against lithocholate-induced liver disease. Thus, failure to sulfate lithocholate could initiate and perpetuate liver damage.

Despite the potential pathophysiological significance of bile salt sulfation, the site and mechanism of sulfate formation have not been elucidated. The present study describes the identification of an enzyme which transfers the sulfate group from 3′-phosphoadenosine-5′-phosphosulfate to bile salts.

Experimental Procedure

Materials

Non-radioactive bile salts were purchased from Calbiochem, La Jolla, California and Supelco Inc., Bellefonte, Pennsylvania. [24-^{14}C]taurolithocholate and sodium [^{35}S]sulfate were purchased from California Bionuclear Co., and New England Nuclear, respectively. Adenosine-3′,5′-diphosphate, estrone sulfate and proteins used for calibration of Sephadex G-100 column chromatography were obtained from Sigma Chemical Company. DEAE-Sephadex A-50 and Sephadex G-100 were purchased from Pharmacia, Piscataway, New Jersey.

Methods

Preparation of 3′-phosphoadenosine-5′-phosphosulfate 3′-phosphoadenosine-5′-phosphosulfate was prepared according to the method of Gregory and Lipmann [8] with the exception that 3′-phosphoadenosine-5′-phosphosulfate was separated from other nucleotides by paper electrophoresis according to the method of Adams and Poulos [9]. Radioactive 3′-phosphoadenosine-5′-phosphosulfate was synthesized with $Na_2{}^{35}SO_4$.

Preparation of enzyme. Cytosols of rat brain, heart, liver, spleen and kidney were prepared by the procedure of Schneider [10]. The minced tissues were homogenized in a cold sucrose (0.25 M) solution with a ratio of 1 : 5 (w/v) utilizing a Potter-Elvehjem homogenizer. The supernates were obtained by cen-

trifuging the homogenates at 105 000 × *g* for 60 min. The clear supernates were used for enzyme studies.

Assay of enzyme activities. The enzyme activity was assayed with a reaction mixture containing 5 μmol of sodium phosphate buffer (pH 6.5), 50 nmol of $MgCl_2$, 7.5 nmol 3′-phosphoadenosine-5′-phosphosulfate, $2—5 \cdot 10^6$ cpm 3′-phosphoadenosine-5′-phospho[^{35}S]sulfate and 10 nmol of taurolithocholate in a total volume of 100 μl. The reaction was initiated by the addition of the enzyme preparation. The mixture was incubated for 10 min at 37° C and terminated by the addition of 0.4 ml of methanol. After standing at 0° C for 5 min, the precipitate was removed by centrifugation and was washed with another 0.4 ml methanol. The combined supernatant solution was evaporated to dryness. The dried residue was dissolved in 25 μl of 60% ethanol and 10 μl of this solution was applied to a thin-layer plate (Adsorbosil-5, Applied Science Lab., Inc.). The plate was developed in a solvent system containing butanol/acetic acid/H_2O (10 : 1 : 1) for 6 h. Sulfated and non-sulfated bile salt standard were visualized by spraying the plate with 10% phosphomolybdic acid. Radioactive areas were located by autoradiography. The radioactive taurolithocholate sulfate was scraped from the plates and counted in a Beckman SL-230 scintillation counter with 10 ml of Bray's scintillant. When assay was performed for estrogen sulfotransferase, estrone (in propylene glycol) was used in place of taurolithocholate, and the thin layer plate (Silica Gel G, Applied Science Lab., Inc.) was developed in a solvent system of chloroform : methanol : H_2O = 130 : 50 : 4 (v/v) for 2 h. One unit of enzyme activity was defined as the amount of enzyme necessary to catalyze the formation of one pmole of bile salt sulfate per minute.

Bile salt analysis. Sulfate esters of bile salts which served as standards were synthesized by the method of Mumma [11]. Solvolysis of bile salt sulfates was accomplished by a modification of the procedure of Burstein and Lieberman [12]. Trifluoracetate substituted methyl cholanates were prepared according to the method of Nair and Garcia [13]. Gas-liquid chromatography of non-sulfated bile acids was performed using 3% OV210 column in a Varian 2100 instrument with flame ionization detector.

Determination of protein. Protein was determined by the method of Lowry et al. [14].

Isoelectrofocusing electrophoresis. Isoelectrofocusing electrophoresis was performed according to the method originally described by Svensson [15] using a 110 ml column (LKB Instruments, Inc.) maintained at 0° C. The pH gradient was established during electrophoresis following the sequential addition of ampholyte solutions (2% w/v) in a 0—47% (w/v) sucrose gradient. The protein solution was applied in the central ampholyte solution. Electrophoresis was initiated at 200 V, increased to 1000 V over a period of 12 h, and continued at this voltage for at least another 12 h for equilibration. Upon completion of the electrophoresis, fractions were collected and assayed for enzyme activity and for pH utilizing a Beckman pH meter.

Results

Identification of product. The incubation of taurolithocholate, 3′-phosphoadenosine-5′-phosphosulfate and rat liver cytosol, results in the formation of

two radioactive products. One of these products has the same R_F value (0.31) as synthetic taurolithocholate (Fig. 1). This compound was not formed when taurolithocholate was not present in the reaction mixture or when boiled enzyme was used. The other product had a R_F value of 0.81 corresponding to cholesterol sulfate. Although we did not investigate this product, this could be the result of the sulfation of endogenous cholesterol. Sulfation of cholesterol has been demonstrated in rat liver [18]. When enzyme preparation was incubated with [24-^{14}C]taurolithocholate and non-radioactive 3′-phosphoadenosine-5′-phosphosulfate, two radioactive compounds were found. One of these compounds corresponded to synthetic taurolithocholate sulfate, while the other had an R_F identical to taurolithocholate. The product which corresponded to the synthetic taurolithocholate sulfate was scraped from the plate, eluted with 95% ethanol, deconjugated with cholylglycine hydrolase and solvolyzed. The resulting bile acids were methylated and derivatized with trifluoracetic anhydride and examined by a gas-liquid chromatography. A single peak which had the same retention time as lithocholate was identified. The taurolithocholate sulfate eluted from the thin layer chromatographic plate was further verified with paper electrophoresis. The radioactive product moved to the same distance as synthetic taurolithocholate sulfate both at pH 2.6 and at pH 7.5.

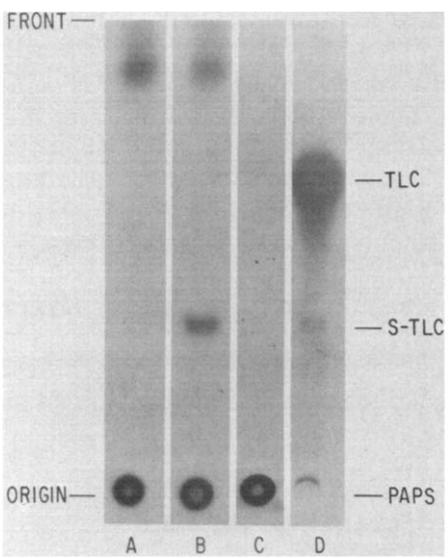

Fig. 1. Radioautogram of the reaction mixture following thin layer chromatography. The complete reaction mixture contained 5 μmol of sodium phosphate buffer (pH 6.5), 50 nmol of $MgCl_2$, 7.5 nmol 3′-phosphoadenosine-5′-phosphosulfate, $3 \cdot 10^6$ cpm 3′-phosphoadenosine-5′-phospho[^{35}S]sulfate, 10 nmol of taurolithocholate (TLC) and 80 μg of protein in a total volume of 0.1 ml. When [^{14}C]taurolithocholate was used, $5 \cdot 10^5$ cpm [^{14}C]taurolithocholate was included in the reaction mixture without labelled 3′-phosphoadenosine-5′-phosphosulfate. Lane A, the reaction mixture without taurolithocholate; Lane B, the complete reaction mixture with 3′-phosphoadenosine-5′-phospho[^{35}S]sulfate and Lane C, the reaction mixture with boiled enzyme. Lane D shows the reaction mixture containing [^{14}C]taurolithocholate and nonradioactive 3′-phosphoadenosine-5′-phosphosulfate. S-TLC, sulfated taurolithocholate.

General properties of the enzyme. In a typical enzyme assay the rate of taurolithocholate sulfation was linear with respect to both enzyme concentration and incubation time. Enzyme activity could be readily detected with as little as 10 μg of cytoplasmic protein in a 0.1 ml reaction mixture. The rates of the reaction with various concentrations of taurolithocholate and 3′-phosphoadenosine-5′-phosphosulfate are shown in Fig. 2. Lineweaver-Burk plots of these data indicate an apparent K_m of $5 \cdot 10^{-5}$ M for taurolithocholate and an apparent K_m for 3′-phosphoadenosine-5′-phosphosulfate of $8 \cdot 10^{-6}$ M.

Table I shows the effect of various compounds on the sulfation of bile salt. The enzyme activity was significantly inhibited by *p*-chloromercuribenzoate and adenosine-3′,5′-diphosphate whereas EDTA and NaF exhibited slight inhibitory effect with a concentration of $1 \cdot 10^{-3}$ M. The enzyme had a pH optimum of 6.5.

Tissue distribution of the enzyme. The survey of the enzyme activity in various rat tissues reveals that liver contains the highest activity (37 units/mg protein). The enzyme activity was not detectable in brain, heart, spleen, intestinal mucosa or red blood cells. However, the enzyme was present in kidney (18 units/mg protein), but the activity was approximately one-half of that found in the liver.

Isolation of the enzyme. All extractions and preparations were conducted at 4°C. Rat liver (4 g) was homogenized in 20 ml of 0.25 M sucrose containing 5 mM Tris/Cl (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol utilizing a Potter-Elvehjem homogenizer with a Teflon pestle. The supernate was obtained by centrifuging the homogenate at 100 000 × *g* for 60 minutes in a Beckman Spinco L2B ultracentrifuge.

Four ml of the resulting solution was applied to a column (1.5 × 20 cm) of DEAE-Sephadex A-50 which had been previously equilibrated with 5 mM Tris/

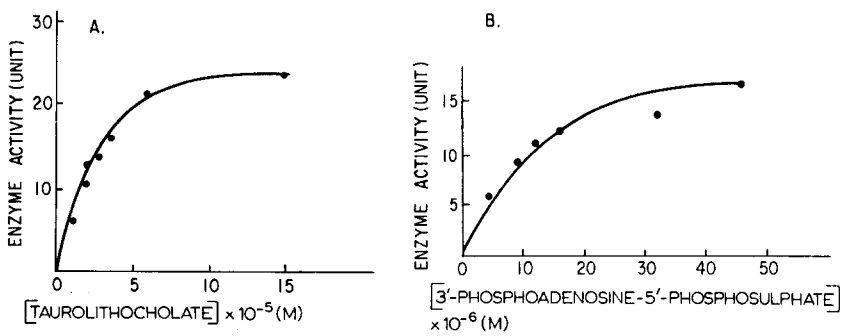

Fig. 2. A. Effect of varying amounts of taurolithocholate on bile salt sulfotransferase. The incubation mixture consisted of 5 μmol sodium phosphate, pH 6.5; 50 nmol $MgCl_2$; 7.5 nmol 3′-phosphoadenosine 5′-phosphosulfate; $1 \cdot 10^6$ cpm [^{35}S]3′-phosphoadenosine-5′-phospho[^{35}S]sulfate; varying concentrations of taurolithocholate and 15 μg of protein in a final volume of 0.1 ml. The Lineweaver-Burk plot of the result gave an apparent K_m value of $5 \cdot 10^{-5}$ M. B. Effect of varying amounts of 3′-phosphoadenosine-5′-phosphosulfate. The reaction mixture contained 5 μmol sodium phosphate buffer, pH 6.5, 50 nmol $MgCl_2$, 10 nmol taurolithocholate, $5 \cdot 10^5$ cpm [^{14}C]taurolithocholate, varying concentrations of 3′-phosphoadenosine-5′-phosphosulfate and 80 μg of protein in a total volume of 0.1 ml. The reaction was incubated at 37°C for 1 h. The Lineweaver-Burk plot of the result gave an apparent K_m value of $8 \cdot 10^{-6}$ M.

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON BILE SALT SULFOTRANSFERASE ACTIVITY

The enzyme activity was determined with a reaction mixture containing 10 nmols taurolithocholate, 5 μmol sodium phosphate buffer (pH 6.5), 50 nmol $MgCl_2$, 7.5 nmol 3′-phosphoadenosine-5′-phosphosulfate, $3 \cdot 10^6$ cpm 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate and 80 μg of rat liver cytosol in a total volume of 0.1 ml. Each reagent was included in the assay mixture with a final concentration of 1 mM. The results are expressed relative to the control without any addition. Other details are described in the text.

Substance	Inhibition (%)
No addition	0
p-chloromercuric benzoate	100
adenosine-3′,5′-diphosphate	100
ATP	45
EDTA	28
NaN_3	33
NaF	27

Cl (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol. The column was washed with 20 ml of the same buffer followed by a linear gradient of sodium chloride (0—0.5 M). The total volume of the gradient was 100 ml. Fractions of 2 ml were collected. A typical chromatogram is shown in Fig. 3. The fractions containing the major enzyme activity were combined and powdered ammonium sulfate was added to 70% saturation. The precipitate was collected with centrifugation and dissolved in 1 ml. of the buffer. The solution was then placed on a Sephadex G-100 column (2 × 90 cm) which had been equilibrated with the same buffer. The enzyme was eluted out after 44 ml of the buffer had passed through the column. The fractions containing the enzyme activity were combined and subjected to isoelectrofocusing electrophoresis. An isoelectrofocusing pattern is presented in Fig. 4. The peak of the enzyme activity corresponded to

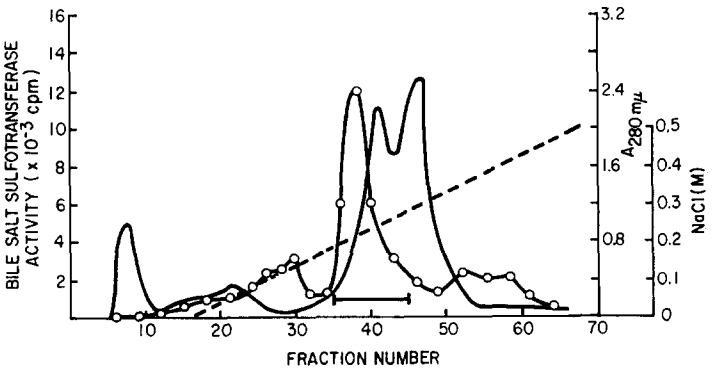

Fig. 3. Chromatography of bile salt sulfotransferase on DEAE-Sephadex A-50. Rat liver cytosol (4 ml) was applied to a column (1.8 × 22cm) of DEAE-Sephadex A-50. The column was equilibrated with 5 mM Tris/Cl buffer (pH 7.5), 1 mM EDTA, and 10 mM β-mercaptoethanol. The column was washed with 20 ml of the same buffer followed by a linear gradient of sodium chloride (0—0.5 M). The total volume of the gradient was 100 ml. Fractions of 2 ml were collected and monitored for protein and enzyme activity. The major active fractions indicated by a bar were pooled. Enzyme activity (o———o), 280 nm (———) and salt concentration (- - - - -).

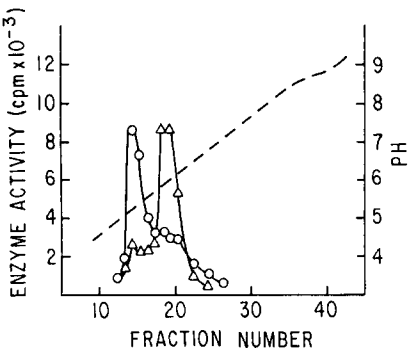

Fig. 4. Isoelectrofocusing electrophoresis of bile salt sulfotransferase. Electrophoresis was performed utilizing ampholyte solutions with a pH range of 3.5—10. Fractions (2 ml) were assayed for pH (- - - - -), bile salt sulfotransferase (○———○) and estrone sulfotransferase (△———△). All other details are described in the text.

a pH value of 5.3. The fractions with the enzyme activity were pooled and dialyzed against the buffer and concentrated to one tenth of the original volume by ultrafiltration. This enzyme preparation was used for the study of substrate specificity. A typical result of the enzyme purification is shown in Table II. An approximate 30-fold purification was achieved by this procedure.

Molecular weight estimation by gel filtration. The molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-100 column according to the method of Andrews [16]. The apparent molecular weight estimated from the known protein standards was 130 000.

Substrate specificity of the enzyme. The rates of sulfation with various bile salts under standard assay conditions were investigated. Rates compared to taurolithocholate as 1.0 were; glycolithocholate 1.2, lithocholate 0.27. It appears that sulfation occurred with both conjugated and unconjuagted bile salts. However the rate of sulfation is higher with conjugated bile salts than with unconjugated. Furthermore, use of taurocholate as substrate for the enzyme reaction gave three products with R_F values of 0.31, 0.25 and 0.16. When taurochenodeoxycholate was used, it gave two products with R_F values of 0.31 and 0.25. Although no standards have been developed for these two bile salts in their various sulfated forms, the data suggest that mono- and disulfation of taurochenodeoxycholate and mono-, di- and trisulfation of tauro-

TABLE II

PURIFICATION OF BILE SALT SULFOTRANSFERASE FROM RAT LIVER

	units/ml	Protein (mg/ml)	units/mg of protein	Total volume (ml)	Total units	Yield (%)	Purification (-fold)
Supernatant	190	8.2	23	4	760	100	1
DEAE-Sephadex A-50	313	2.9	108	2	616	81	5
Sephadex G 100	35	0.1	350	14	490	65	15
Isoelectrofocusing	75	0.1	750	4	300	40	33

cholate occurred in the reaction mixture. Thus, sulfation may take place with both primary and secondary bile salts.

Discussion

The present report describes an enzymatic reaction in the cytosol of rat liver and kidney which catalyzes the sulfation of bile salts. The product of the reaction was identified by its thin layer chromatographic behavior, paper electrophoretic mobility at two pH systems, and its gas-liquid chromatographic retention time after acid solvolysis.

The requirement of metal ions for sulfotransferases has been a matter of controversy. Some investigators have shown increased estrogen sulfotransferase activity in the presence of Mg^{2+} [9], whereas others have failed to show this effect. The discrepancy in these findings may be due to differences in the degree of enzyme purification. In this study we did not find an absolute requirement for metal ions. However, the enzyme was partially inhibited by the chelating agent EDTA.

Preliminary studies of substrate specificity demonstrate that the enzyme reacts with conjugated as well as unconjugated bile salts. However the rate of sulfation is higher with conjugated than with unconjugated species under standard assay condition.

Nose and Lipmann [19] have identified at least three sulfotransferases in the rabbit liver. One of these enzymes catalyzes the transfer of the sulfate group from 3′-phosphoadenosine-5′-phosphosulfate to dehydroepiandrosterone, while the other two enzymes transfer sulfate from 3′-phosphoadenosine-5′-phosphosulfate to phenol and to estrone, respectively. Although we cannot state with certainty that the enzyme responsible for the bile salt sulfation is a specific entity, we have shown that this enzyme has an isoelectric point of 5.3 whereas estrone sulfotransferase has an isoelectric point of 6.1 (Fig. 4). Furthermore, this enzyme has a molecular weight of 130 000, is considerably larger than estrogen sulfotransferase which has a molecular weight of 74 000 [22]. In addition since there is a difference in stereo-configuration and functional groups between bile salts and neutral steroids, this is indicative of a different enzyme than the previously described steroid sulfotransferases.

The site of steroid sulfation is generally believed to occur mainly in the liver, although sulfation of neutral steroids has been demonstrated in the intestinal wall [20] as well as the adrenal gland [21]. A survey of rat tissues revealed that bile salt sulfation occurred in both rat liver and kidney, but not detectable in other tissues examined. Steil et al. [4] have shown that significant amounts of sulfated bile salts were excreted in urine of a patient with intrahepatic biliary atresia and liver diseases. Similar data were reported by Makino et al. [5]. Our finding of bile salt sulfation in rat kidney suggests that urinary bile salt sulfate may arise in part from the de novo synthesis in the kidney. What role the kidney plays in the bile salt metabolism is currently under investigation.

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