Human liver steroid sulphotransferase sulphates bile acids

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The sulphation of bile acids is an important pathway for the detoxification and elimination of bile acids during cholestatic liver disease. A dehydroepiandrosterone (DHEA) sulphotransferase has been purified from male and female human liver cytosol using DEAE-Sepharose CL-6B and adenosine 3',5'-diphosphate-agarose affinity chromatography [Falany, Vazquez & Kalb (1989) *Biochem. J.* **260**, 641–646]. Results in the present paper show that the DHEA sulphotransferase, purified to homogeneity, is also reactive towards bile acids, including lithocholic acid and 6-hydroxylated bile acids, as well as 3-hydroxylated short-chain bile acids. The highest activity towards bile acids was observed with lithocholic acid (54.3 ± 3.6 nmol/min per mg of protein); of the substrates tested, the lowest activity was detected with hyodeoxycholic acid (4.2 ± 0.01 nmol/min per mg of protein). The apparent K_m values for the enzyme are 1.5 ± 0.31 μ M for lithocholic acid and 4.2 ± 0.73 μ M for tauroolithocholic acid. Lithocholic acid also competitively inhibits DHEA sulphation by the purified sulphotransferase (K_i 1.4 μ M). No evidence was found for the formation of bile acid sulphates by sulphotransferases different from the DHEA sulphotransferase during purification work. The above results suggest that a single steroid sulphotransferase with broad specificity encompassing neutral steroids and bile acids exists in human liver.

INTRODUCTION

Biliary and urinary excretion of endogenous or exogenous toxic compounds usually requires biotransformation to more polar metabolites. Generally, two major biochemical mechanisms have evolved for the reduction of hydrophobicity and toxicity of these compounds: oxidation (mainly hydroxylation) and conjugation (mainly glucuronidation, sulphation, and glutathione conjugation). Often, hydroxylation (a Phase I detoxification reaction) is followed by conjugation (Phase II reaction). Many, but not all, of the enzymes catalysing the above reaction are localized in the liver.

Sulphotransferases catalyse the transfer of a sulphate group from 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to a wide variety of xenobiotics, as well as to endogenous compounds such as steroid hormones and bile acids (for a review, see [1]). Sulphotransferase activities of various specificities and functions occur in many human tissues, including liver [2–4], small intestine [5] and adrenals [6,7]. These enzymes may exhibit the same marked polymorphism as that seen in animal sulphotransferases [8–11]. Among the different forms of human sulphotransferases already described, there are two forms which sulphate bile acids [2,3].

Palmer [12] was the first to demonstrate the formation and the urinary excretion of bile acid sulphates in humans. Subsequently, bile acid sulphation has been shown to be an important metabolic step in human bile acid metabolism. The formation of bile acid sulphates was more fully confirmed by studies on cholestatic and cirrhotic livers [13,14] and on patients with biliary atresia [15]. It has been well established that, in the absence of pathological conditions, the biliary pathway is quantitatively the main route for secretion of sulphated bile acids in man [14–16]. These 'in vivo' data were confirmed by 'in vitro' measurements of bile acid sulphotransferase activity in hepatic, adrenal and intestinal

cytosols [2,3,5,6,17,18]. Bile acid sulphation is considered to be important for the elimination of potentially toxic bile acids [19].

Only two attempts to purify bile acid sulphotransferase from human liver have been reported. A human liver enzyme catalysing the transfer of a sulphate group from PAPS to the hydroxy function of bile acids was originally described by Loof & Hjerten [2]. This protein, 3'-phosphoadenylylsulphate:tauroolithocholate sulphotransferase (bile-salt sulphotransferase, EC 2.8.2.14), was also active toward a variety of other steroids, including oestrone and dehydroepiandrosterone (DHEA). In addition, the enzyme exhibited activity towards phenols. The preparation yielded a single band on SDS/PAGE; its M_r was not reported. The sulphotransferase had a pI of 5.5 and a broad pH optimum between 7.5 and 9. The purification procedure suffered from a very low recovery of bile acid sulphotransferase activity, suggesting that enzyme inactivation was a major problem. No other liver steroid or bile-acid sulphotransferase activity was detected during this procedure. Chen & Segel [3] used a different purification scheme, which included taurocholate-agarose affinity chromatography. The purification resulted in a preparation of M_r 67000 and pI 5.2. The final preparation had a high specific activity and represented a 760-fold purification from the cytosol with an overall yield of 15%. The purified enzyme exhibited a pH optimum of 6.5, with 50% activity at pH 5.8 or pH 7.5. This protein differed from Loof & Hjerten's [2] preparation in its relative specificity for unconjugated and conjugated bile acids as the sulphate acceptors, and exhibited no activity with a variety of other steroids and phenols. Both Loof & Hjerten [2] and Chen & Segel [3] purified their activities from normal male livers.

Recently, Falany *et al.* [20] demonstrated that human liver steroid sulphotransferase could be purified to homogeneity by DEAE-Sepharose CL-6B and adenosine 3',5'-diphosphate-

Abbreviations used: DHEA, dehydroepiandrosterone (3 β -hydroxyandrost-5-ene-17-one); PAPS, 3'-phosphoadenosine 5'-phosphosulphate; LA, lithocholic acid (3 α -hydroxy-5 β -cholan-24-oic acid); 3 α ,6 α -, 3 α ,6 β - and 3 α ,7 α -diOH, hyo-, muri- and cheno-deoxycholic acid; 3 α ,12 α -diOH, deoxycholic acid; 3 α ,7 α ,12 α -triOH, cholic acid.

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agarose affinity chromatography. During affinity chromatography, DHEA sulphation activity could be resolved from the two forms of phenol sulphotransferase present in human liver, confirming that, in human liver, DHEA sulphotransferase is a unique form of sulphotransferase. The purified enzyme was most active toward DHEA, but was capable of conjugating a number of other steroids, including testosterone and oestrone. The final preparation had a specific activity toward DHEA of 133 nmol/min per mg of protein, representing a 600-fold purification from the cytosol fraction with an overall yield of 8%. The enzymically active form of the enzyme has M_r 68 000–70 000 and a pH optimum of 7.5. After SDS/PAGE the pure enzyme had a subunit M_r of approx. 35 000. This hepatic DHEA sulphotransferase is apparently different from the partially purified bile-acid sulphotransferase described by Chen & Segel [3], as the latter enzyme displayed no activity toward testosterone and oestrone, both of which are good substrates for the DHEA sulphotransferase. The DHEA sulphotransferase is also different from the enzyme reported by Loof & Hjerten [2], since the DHEA sulphotransferase does not catalyse sulphation of phenols.

The purpose of the present study was (a) to investigate the reactivity of the purified human steroid (DHEA) sulphotransferase towards bile acid substrates, (b) to expand the 'in vitro' studies of sulphation of bile acids by human liver cytosol and purified DHEA sulphotransferase using representative bile acids having various configurations of the molecule, numbers of hydroxy groups and side-chain lengths, and (c) to compare the sulphation of steroid and bile acid substrates in individual normal human liver cytosols and purified preparations of DHEA sulphotransferase obtained from donors of different sexes and ages. The characterization of human liver steroid sulphotransferase with bile acid substrates may provide an insight into the polymorphism of sulphotransferases in human liver, as well as help in the elucidation of the role of hepatic sulphation in steroid and bile acid metabolism.

EXPERIMENTAL

Materials

Short-chain bile acids, unlabelled or ^3H -labelled, were purchased or synthesized as described previously [21,25], and checked for chemical purity by t.l.c. and g.l.c. [*carboxy*- ^{14}C]-Lithocholic acid (59 mCi/mmol), [*carboxy*- ^{14}C]deoxycholic acid (58 mCi/mmol) and dehydro[1,2,6,7- ^3H]epiandrosterone (^3H]DHEA) (60–100 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). [2,4- ^3H]Cholic acid (25.0 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). [3 β - ^3H]3 α -Hydroxy-5 β -cholanoic acid [tritiated lithocholic acid (LA)] was prepared by reduction of methyl 3-oxo-5 β -cholanoate with NaB^3H_4 (360.0 mCi/mmol), followed by separation of the resulting 3-epimers and hydrolysis of the methyl ester, as described previously for other bile acids [21]. Tauro[^3H]lithocholic acid was synthesized from tritiated lithocholic acid by a published procedure [23]. ^3H -labelled hyo- (3 α ,6 α -diOH), muri- (3 α ,6 β -diOH) and cheno- (3 α ,7 α -diOH) deoxycholic acid were synthesized as described by Radomska *et al.* [21] for ^3H -labelled short-chain bile acids; underivatized 3 α -hydroxy-6-oxo- and 3 α -hydroxy-7-oxo-5 β -cholanoic acids (Steraloids, Wilton, NH, U.S.A.) were reduced with NaB^3H_4 (360.0 mCi/mmol), and the resulting α and β epimers in positions 6 and 7 were separated by preparative h.p.l.c. Preparative reverse-phase h.p.l.c. was carried out using a μ Bondapak C_{18} column (0.78 cm $\times$ 30 cm; Waters, Milford, MA, U.S.A.) with a CM400 gradient pump (Milton Roy/LDC, Riviera Beach, FL, U.S.A.) and a model 1840 variable-wavelength detector (ISCO, Lincoln,

NE, U.S.A.) set at 205 nm. The elution protocol was as follows: 0–40 min, methanol in 5 mM potassium phosphate, pH 5.0; 40–55 min, linear gradient of 75–90% methanol in the above buffer; 55–70 min, 90% methanol in the above buffer; 70–80 min, linear gradient of 90–100% methanol in the above buffer; after 80 min, 100% methanol. The solvent flow rate was 2 ml/min, and 2 ml fractions were collected. Radioactive peaks were pooled, concentrated, acidified with 1 M-HCl, and extracted with ethyl acetate. PAPS and PAPS-agarose were purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Steroids, Brij 58, DEAE-Sephacrose CL-6B and other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Analytical and preparative t.l.c. was carried out on LK5 plates (Whatman Chemical Separation Inc., Clifton, NJ, U.S.A.) or Si250-PA(19C) plates (J. T. Baker, Phillipsburg, NJ, U.S.A.). The monosulphate esters of bile acids were obtained from Sigma Chemical Co. or synthesized from appropriately protected bile acids by a facile reaction using the sulphur trioxide-triethylamine complex [24].

Human liver samples

Human liver samples were obtained from the Organ Procurement Program, University of Rochester, after donation of other organs for transplantation. Table 1 contains a short description of the donors. Cytosol from the individual livers was prepared as previously described [20].

Purification of DHEA sulphotransferase

DHEA sulphotransferase was purified from cytosol prepared from individual human livers using DEAE-Sephacrose CL-6B and adenosine 3',5'-diphosphate-agarose affinity chromatography as described previously [34]. Generally, 500–700 mg of cytosolic protein was applied to a DEAE-Sephacrose CL-6B column (2 cm $\times$ 16 cm) pre-equilibrated with 10 mM-triethanolamine, pH 7.5, containing 1.5 mM-dithiothreitol and 10% (v/v) glycerol (Buffer A). The column was then washed with 4 bed vol. of Buffer A, followed by 100 ml of the same buffer containing 100 mM-NaCl. Elution of sulphotransferase activities was carried out with a 100–225 mM-NaCl gradient (total volume 600 ml) in Buffer A. Fractions (6.5 ml each) were collected and aliquots of the fractions were assayed for DHEA sulphotransferase activity. Fractions containing DHEA sulphotransferase activity were pooled and concentrated by ultrafiltration (Diaflo PM 30 membrane) to approx. 8 ml. The concentrated pool was applied to an adenosine 3',5'-diphosphate-agarose affinity column (0.7 cm $\times$ 10 cm) previously equilibrated with Buffer A. The column was washed with 6–7 bed vol. of Buffer A containing 50 mM-NaCl. Sulphotransferase activity was eluted from the

Table 1. Characteristics of human liver samples

Liver no.	Sex	Age (years)	Cause of death
2	Female	6	Anoxia
3	Female	56	Cerebral bleed
4	Female	55	Cerebral bleed
5	Male	34	Cerebral bleed
6	Male	56	Cerebral bleed
7*	Female	14	Anoxia
8	Male	41	Cerebral bleed
9	Female	41	Cerebral bleed
10	Female	66	Cerebral bleed

* Patient 7 was undergoing phenobarbital therapy for the control of organic seizures.

affinity column with a linear gradient (30 ml total volume) of 0–15 μM -PAPS in Buffer A containing 50 mM-NaCl.

Enzyme assays

Bile acid sulphotransferase activity was measured with both conjugated and unconjugated bile acids as substrates. Bile acids were prepared in the form of mixed micelles with Brij 58 (final concentration of detergent in the reaction mixture: 0.006%), as described in detail in [25]. Briefly, a 1 mM stock solution of bile acid was prepared as follows: 1 μmol of bile acid of the desired specific radioactivity (for lithocholic acid, usually carrier-free, i.e. 57 Ci/mol) was dissolved in methanol and mixed with 120 μl of aq. 1% (w/v) Brij 58 and 20 μl of 0.1 M-NaOH. The mixture was evaporated at 40 °C under a stream of N_2 , and 1 ml of water was added to the dry residue. Brief sonication was required for clarification. Alternatively, bile acids and/or DHEA were introduced into the reaction mixture in propylene glycol. The stock solution was prepared by overlaying 1 ml of propylene glycol with a solution of bile acid or sterol in methanol, and evaporation of the methanol under a stream of N_2 . The resulting stock of a desired concentration of substrate was used in the assay to give a final concentration of propylene glycol in the reaction mixture of 0.5%. Both methods gave identical results, and the micelle method was used in most experiments reported in the present paper. Bile acid sulphotransferase activity was assayed in 60 μl of 100 mM-Tris/HCl buffer (pH 7.5)/10 mM- MgCl_2 /20 μM -PAPS, containing 5 μl of purified enzyme preparation (approx. 0.1–0.2 μg of pure protein). Sulphotransferase activity toward various bile acids was assayed at substrate concentrations listed with the particular experiments. After 10 min at 37 °C, reactions were stopped by addition of 20 μl of ethanol, and 60 μl of the mixture was directly applied to the preadsorbent layer of 10-channelled preparative silica-gel t.l.c. plates. The sulphated bile acids and substrate that had not reacted were separated by t.l.c. in chloroform/methanol/acetic acid/water (65:24:10:5, by vol.) or, for additional product identification, in n-butanol/acetic acid/water (10:1:1, by vol.). Under the above conditions, no more than 10% of the bile acid substrate was converted into the sulphated product. In contrast, for screening of the column fractions, 15 μl of each fraction was used, and the reaction time was 15 min. This led to a total depletion of substrate in peak fraction. Therefore the true activity of the fraction is higher than shown in Figs. 2 and 3 (below). This method, designed to maximize sensitivity, was used only to locate the peak; for subsequent kinetic measurements, the smaller amount of protein and shorter reaction time, as described above, were used.

Inhibition of LA sulphation by DHEA was measured as described above, except that variable concentrations of labelled LA (0.25–20 μM) were used in conjunction with at least four concentrations of DHEA (in propylene glycol). Specific activities of enzymes are expressed as nmol/min per mg of protein; where applicable, means $\pm$ S.D. are reported. Radioactive compounds were localized on t.l.c. plates by scanning with a Tracemaster 20 windowless gas-flow counter (Berthold Analytical Instruments, Nashua, NH, U.S.A.), and/or by autoradiography at –80 °C. Before autoradiography, t.l.c. plates with tritium-labelled compounds were sprayed with En^hance (du Pont–NEN, Boston, MA, U.S.A.). Zones of the silica gel corresponding to the sulphate-containing compound and substrate that had not reacted were scraped directly into scintillation vials, and the radioactivity was determined by scintillation spectrometry. In the solvent system chloroform/methanol/acetic acid/water (65:24:10:5, by vol.), used routinely for all assays, the radioactive bile acid substrates were clearly separated from the bile acid sulphates. Sulphate metabolites were identified by their co-

chromatography with authentic standards on silica-gel thin-layer plates developed in two different solvent systems. For LA and other monohydroxylated bile acid substrates, a single radioactive reaction product with a mobility equal to the corresponding 3-monosulphate standard was found. The same was observed in the case of polyhydroxylated bile acids; however, the position of the sulphate residue in the molecule has not been determined. DHEA sulphotransferase reaction mixtures routinely contained 50 mM-Tris/HCl (pH 7.5)/10 mM- MgCl_2 /3 μM -[³H]DHEA (sp. radioactivity 0.05 $\mu\text{Ci/nmol}$) and 20 μM -PAPS. Reactions were terminated by the addition of 3.0 ml of chloroform, and 0.25 ml of 0.25 M-Tris/HCl, pH 8.7, was added to alkalize the solution. The reaction tubes were then vortex-mixed twice for 15 s and centrifuged at 600 *g* for 5 min to separate the aqueous and organic phases. Synthesis of DHEA sulphate (which remains in the aqueous phase) was then determined by scintillation counting of an aliquot of the aqueous phase. Phenol (with *p*-nitrophenol as the substrate) sulphotransferase activity was measured as previously described [26].

Other methods

SDS/PAGE was performed by the method of Laemmli [27], in a Bio-Rad Protean II unit, and protein bands were revealed by Coomassie Blue staining. Protein concentrations were determined by the Coomassie Blue dye-binding method (Bio-Rad) described originally by Bradford [28], with BSA as standard.

Data processing

Maximal velocities and Michaelis constants were calculated using a numerical version of the direct-linear-plot method [29–31], robust non-linear curve-fitting [32] or the Eadie–Hofstee transformation. K_i values were determined from secondary plots of slope versus inhibitor concentration of data obtained from the primary double-reciprocal plots [33].

RESULTS

Bile acid sulphotransferase activity in different human livers

One of the goals of the present study was to determine whether DHEA sulphotransferase is also responsible for the sulphation of bile acids in human liver. Sulphotransferase activity towards LA and DHEA (Fig. 1) was assayed in cytosolic preparations from several human livers (Table 1). In the individual livers, DHEA sulphotransferase activity paralleled LA sulphotransferase activity (correlation coefficient, *r*, 0.88). The latter activity varied between 0.16 and 0.4 nmol/min per mg of cytosol protein. No obvious correlation of sulphotransferase activity with the age or sex of the liver donors was found. The lowest sulphotransferase activity was found in liver 4 (55-year-old female), and the highest in liver 6 (56-year-old male). Liver 2 (6-year-old female), liver 6 (56-year-old male) and liver 11 (49-year-old female) (results not shown) were used for purification of DHEA sulphotransferase and enzyme-characterization studies.

Enzyme purification

DHEA sulphotransferase was purified as described previously [34]. Briefly, human liver cytosol was chromatographed on DEAE-Sepharose CL-6B (Fig. 2). Fractions containing DHEA- and LA-sulphating activity eluted at about 0.15 M-NaCl. These fractions were pooled, concentrated, and subjected to adenosine 3',5'-diphosphate-agarose affinity chromatography (Fig. 3). About 90% of the DHEA- and LA-sulphating activity was adsorbed on to the affinity column. After removal of non-specifically adsorbed protein by washing of the affinity column with Buffer A containing 50 mM-NaCl, the DHEA and LA

Fig. 1. DHEA and LA sulphotransferase activity in cytosol preparations from different human livers

Cytosol was prepared as described in the Experimental section; results for individual livers are presented in Table 1. Reactions contained $5 \mu\text{M}$ - ^{14}C LA (in the form of Brij 58 micelles; see the Experimental section) or $3 \mu\text{M}$ - ^3H DHEA in propylene glycol (0.5% final concn.), $30 \mu\text{M}$ -PAPS and $35 \mu\text{g}$ of cytosolic protein. Results for LA sulphation ($\square$) are given as means $\pm$ S.D. ($n = 4$), with n denoting the number of separate assays; results for DHEA sulphation ($\blacksquare$) are means for two experiments.

Fig. 2. DEAE-Sepharose CL-6B chromatography

Cytosol obtained from liver 6 (700 mg of protein) was applied to the anion-exchange column (2.5 cm $\times$ 10 cm) equilibrated with 10 mM-triethanolamine (pH 7.5)/1.5 mM-dithiothreitol/10% (v/v) glycerol. The column was washed with 200 ml of this equilibration buffer, followed by 100 ml of 100 mM-NaCl in the same buffer. Sulphotransferase activity was then eluted with a linear gradient of NaCl (100–225 mM-NaCl) in the above buffer in a total volume of 600 ml, and 6.5 ml fractions were collected. Bile acid sulphotransferase activity was assayed in the fractions as described in the Experimental section at $25 \mu\text{M}$ unlabelled PAPS, $5 \mu\text{M}$ - ^{14}C LA ($\blacksquare$), or $3 \mu\text{M}$ - ^3H DHEA ($\square$), $15 \mu\text{l}$ of the fraction being used in each assay.

sulphotransferase activities were specifically eluted with a gradient (0–20 μM) of PAPS. On both chromatography columns, the sulphotransferase activities towards DHEA and LA were found as a single peak. In both cases, the elution profile of activity capable of sulphation of LA matched the elution profile of

Fig. 3. Adenosine 3',5'-diphosphate-agarose affinity chromatography

Pooled fractions containing sulphotransferase activity from DEAE-Sepharose CL-6B chromatography were applied to an adenosine 3',5'-diphosphate-agarose column (0.7 cm $\times$ 10 cm). Non-adsorbed proteins were removed with 10 mM-triethanolamine, pH 7.5, containing 1.5 mM-dithiothreitol, 10% glycerol and 50 mM-NaCl. Sulphotransferase activity was then specifically eluted with a gradient of PAPS (0–15 μM) in the same buffer; 2 ml fractions were collected. LA ($\blacksquare$) and DHEA ($\square$) sulphotransferase activities were assayed as described in the legend to Fig. 2.

DHEA sulphotransferase activity, but not the phenol (*p*-nitrophenol) sulphotransferase (results not shown) [20].

Kinetic properties of bile acid sulphation by human DHEA sulphotransferase

The assays of bile acid sulphotransferase activity were carried out in the presence of the detergent Brij 58. The presence of small amounts of detergent is needed to solubilize the hydrophobic monohydroxylated bile acid substrates; at the concentration used (final concn. 0.006%), the detergent does not inhibit enzyme activity. By this modification of previously published methods, in which an alcoholic solution of the bile acid was evaporated to dryness before buffer addition, a specific activity of bile acid sulphotransferase for monohydroxylated bile acids (40–50 nmol per min/mg) was achieved that is appreciably higher than that reported previously [2,3], probably because of an improved availability of the substrate. The incubation volume was $60 \mu\text{l}$; thus, the entire incubation mixture could be transferred on to a t.l.c. plate, dried, and the plate developed. The above simple modification permitted rapid and efficient separation of the bile acid substrates that had not reacted from their sulphates by preparative t.l.c. and, in the same step, product identification. DHEA sulphotransferase with LA and DHEA as substrates exhibited a broad pH range (pH 7.5–8.8) and was maximally active in Tris buffer over the pH range 7.7–8.0. In contrast with previous reports of human liver bile acid sulphotransferase [3], the present enzyme preparation did not catalyse sulphation of LA below pH 6 (Fig. 4).

Sulphation of LA and tauro lithocholic acid was measured at a saturating concentration of PAPS (30 μM) [20] with various concentrations of the respective bile acid substrate. The reaction followed Michaelis–Menten-type kinetics, and kinetic parameters calculated by robust non-linear curve-fitting [32], an algorithm based on the direct linear plot [29–31], or the Eadie–Hofstee transformation, were in close agreement; the Michaelis constants

Fig. 4. Effect of pH on LA sulphation by cytosol and purified enzyme

Reactions (at 10 μ M-substrate) were carried out as described in the Experimental section, except that the buffer was 0.1 M-Mes for pH 5.5 and 6.0, 0.1 M-Hepes and/or 0.1 M-Tris for 7.5, 8.0 and 8.5, and 0.1 M-Tris for pH 9.0. The pH values represent the final pH of the reaction mixture. Cytosol ($\square$) and purified enzyme ($\blacksquare$) from human liver 2 were used. An identical profile was obtained for liver 6 (not shown).

were 1.5 and 4.2 μ M for LA and tauroolithocholic acid respectively. Those apparent Michaelis constants were in good agreement with published values [2,3], indicating that observed apparent K_m values are relatively independent of the method of substrate preparation and pH value. However, these factors have a significant effect on the reaction rate, as reflected in the V_{max} values. Substrate inhibition of LA sulphation was observed, even though it was somewhat variable. Usually, concentrations exceeding 20 μ M were necessary for inhibition, in contrast with DHEA and other natural steroids, which exhibited substrate inhibition at concentrations as low as 2.5 μ M [20]. No substrate inhibition was found with tauroolithocholic acid. The utilization ratios (V_{max}/K_m) were calculated for two of the bile acid substrates and DHEA and are shown in Table 2. DHEA is a competitive inhibitor of LA and tauroolithocholic acid sulphation catalysed by purified human bile acid sulphotransferase. The apparent K_i value for LA, 1.5 μ M, is similar to its apparent K_m as a substrate (Table 2).

Substrate specificity of human sulphotransferase

All bile acids which were sulphated by human liver cytosol were also substrates for the purified liver DHEA sulpho-

Table 2. Apparent Michaelis constants of purified sulphotransferase for steroid and bile acids

Apparent K_m and V_{max} were determined at 0.025 to 20 μ M-bile acid and a constant concentration (30 μ M) of PAPS. Results are means $\pm$ S.D. ($n = 3-6$) of determinations carried out on enzyme preparations from liver 2 and liver 6. Abbreviation: TLA, tauroolithocholic acid.

Substrate	Apparent K_m (μ M)	V_{max} . (nmol/min per mg of protein)	Utilization ratio (V_{max}/K_m)
DHEA	1.6 $\pm$ 0.1	143.0 $\pm$ 9.0	84.4
LA	1.5 $\pm$ 0.31	94.4 $\pm$ 7.6	56.6
TLA	4.2 $\pm$ 0.73	59.6 $\pm$ 2.47	14.2

*Data from [20] and the present work.

Fig. 5. Specific activity of hepatic human sulphotransferase toward conventional and 6-hydroxylated bile acids

The results shown were obtained with cytosol (hatched bars) and purified enzyme (filled bars) from human liver 6. Results are plotted as means $\pm$ S.D. ($n = 4$), with n denoting the number of separate experiments, each done in duplicate. The substrate concentration was 10 μ M for all bile acids. Bile acid sulphation reactions (with 35 μ g of cytosolic protein and approx. 0.2 μ g of protein for purified enzyme) were carried out as described in the Experimental section. Further abbreviation used: TLA, tauroolithocholic acid [*N*-(3 α -hydroxy-5 β -cholan-24-oyl)taurine].

transferase. The quantitative pattern of specific activities of the sulphation reaction for different bile acids was similar for liver cytosol and the enzyme purified from the same liver (Figs. 5 and 6). Both cytosolic sulphotransferase activity and purified DHEA sulphotransferase very effectively conjugated monohydroxylated bile acids with a 3 α -hydroxy group; LA has proven to be the best substrate of all bile acids tested so far. The monohydroxylated short-chain bile acid $C_{21}\alpha\beta$ (see Fig. 6) showed the second

Fig. 6. Specific activity of hepatic human sulphotransferase toward short-chain C_{20-23} bile acids and LAs

Bile acid sulphation was performed as described in the legend to Fig. 5. Abbreviations: $C_{20}\alpha\beta$, 3 α -hydroxy-5 β -androstane-17 β -carboxylic acid; $C_{21}\alpha\beta$, 3 α -hydroxy-5 β -pregnan-21-oic acid; $C_{22}\alpha\beta$, 3 α -hydroxy-23,24-bisnor-5 β -cholan-22-oic acid; $C_{23}\alpha\beta$, 3 α -hydroxy-24-nor-5 β -cholan-23-oic acid; $C_{20}\beta\beta$, 3 β -hydroxy-5 β -androstane-17 β -carboxylic acid; $C_{24}\beta\beta$, isolithocholic acid, 3 β -hydroxy-5 β -cholan-24-oic acid.

highest rate of sulphation, with the rest of the 3 α -hydroxylated short-chain bile acids being sulphated at a 2–3-fold lower rates. Isolithocholic acid and C₂₀ $\beta\beta$ (see Fig. 6), which possess a 3 β -hydroxy group, are also very good substrates. As Fig. 5 shows, the cytosol and the purified sulphotransferase catalyse the sulphation of deoxycholic acid (3 α ,12 α -diOH), but their activities toward 3 α ,7 α -diOH and cholic acid (3 α ,7 α ,12 α -triOH) are very low. 3 α ,6 β -diOH, a bile acid which possesses a 6 β -hydroxy group, exhibited a relatively high rate of sulphation (similar to 3 α ,12 α -diOH) with both cytosol and purified enzyme. However, 3 α ,6 α -diOH (a 6 α -hydroxylated compound) was among the least active substrates of all bile acids studied. The position of sulphate groups in the dihydroxylated bile acids (3,6,7 or all) has not been determined.

DISCUSSION

A considerable body of information is now available on bile acid/steroid sulphotransferase multiplicity in experimental animals [9–11,34,35], but only a few attempts have been made to purify and characterize bile acid sulphotransferases from human tissues. The purpose of the present work is a closer characterization of bile acid sulphation in human liver.

Four lines of evidence presented here indicate that the formation of bile acid and steroid sulphates is catalysed by a single sulphotransferase form: (i) the activity profiles of nine different human livers for bile acid (LA) and steroid (DHEA) sulphation are identical; (ii) the elution profile of activity capable of bile acid sulphation matched the elution profile of DHEA sulphotransferase on ion-exchange and affinity chromatography, and the sulphotransferase activities toward DHEA and LA were found as a single peak; (iii) purified steroid sulphotransferase was able to conjugate all the bile acids that are sulphated in the cytosol, and the ratio of the specific activity of the purified enzyme to that of cytosol was the same for all substrates; (iv) LA inhibited the sulphation of DHEA by the purified steroid sulphotransferase in a competitive manner.

Sulphotransferase activity in cytosol prepared from liver samples of nine donors of different ages and sexes was investigated. These livers were found to exhibit large (up to 260%) inter-individual variations. Bile acid sulphotransferase activity was not correlated with age or sex, in contrast with rat and hamster, where steroid sulphotransferase activity, including bile acid sulphotransferase, appears to be significantly influenced by sex hormones [8–11,36]; female rat and hamster livers contain 2–6 times the activity observed in male livers [36]. Our present observations confirm those of Loof & Nyberg [18], who have reported large variations of bile acid sulphotransferase levels in percutaneous liver biopsy specimens, and did not observe a sex difference with respect to human bile acid sulphotransferase activity in male and female livers [5].

The ratio of activities for DHEA and LA were very similar in all nine livers studied (Fig. 1). This provided the first strong indication that DHEA sulphotransferase is responsible for the formation of sulphates of bile acids. A further confirmation of this conclusion was reached by examining the behaviour of these activities during purification. On both ion-exchange and affinity-chromatography columns, the sulphotransferase activities towards DHEA and LA were found as a single peak. In both cases the elution profile of enzyme activity capable of the sulphation of LA matched the elution profile of DHEA sulphotransferase, but not that of phenol (*p*-nitrophenol) sulphotransferase. Elution profiles were similar for all livers studied, regardless of age and sex.

While the purification procedure was monitored, activity of the bile acid sulphotransferase was assayed at both pH 7.5, the

pH optimum for steroid [20] and LA (Fig. 4) sulphation reactions, and 6.5, the pH value used by Chen & Segel [3]. Elution profiles were identical, but the activity was 2.6–3.3-fold higher at pH 7.5 than at pH 6.5. No other activity specific for bile acids has been observed. These results demonstrate that the steroid sulphotransferase purified to apparent homogeneity by Falany *et al.* [20] is the same protein that sulphates bile acids. The final preparation had a specific activity with LA of 40–52 nmol/min per mg of purified enzyme protein, which is 3- or 30-fold higher than the values reported by Chen & Segel [3] and Loof & Hjerten [2], respectively. The very low specific activities of these latter preparations suggest that enzyme inactivation, assay conditions (such as pH) or availability of substrate were potential problems.

The apparent Michaelis constants for LA and conjugated LA (tauro lithocholic acid) were in the range 1–4 μ M, which is the same order of magnitude as the K_m for DHEA (1 μ M), the prototypical substrate of DHEA sulphotransferase. The utilization ratio (ratio of V_{max} to K_m) for LA was between the ratio for DHEA and that for tauro lithocholic acid. It can thus be concluded that unconjugated monohydroxylated bile acids are better substrates for DHEA sulphotransferase than conjugated ones, but not as good as DHEA. Competition between DHEA and LA (or tauro lithocholic acid) for the purified sulphotransferase serves as the most direct proof that it is the DHEA sulphotransferase, rather than a different, hypothetical, enzyme that co-purifies with it, that is responsible for the sulphation of bile acids.

A comparison of the pattern of sulphation of bile acids by human cytosols and by the purified sulphotransferase yields interesting insights concerning the number of sulphotransferase isoenzymes present in human liver. For all short-chain, conventional and 6-hydroxylated bile acids, the ratio of the specific activity of the purified enzyme to that of cytosol is the same (approx. 100) and can be treated as the apparent purification factor of the enzyme. The fact that, with all bile acid substrates, the same enrichment was obtained can be best explained by assuming that all bile acids are sulphated exclusively by the same sulphotransferase.

Bile acids can occur as two epimers, 3 α - and 3 β -hydroxy, with the former being prevalent in biological materials. The specificity of human sulphotransferase for 3 α and 3 β epimers has been studied for several conventional (C₂₄) and short-chain (C_{20–23}) monohydroxylated bile acids (Fig. 5). In contrast with UDP-glucuronosyltransferases, the other Phase II conjugation enzymes that act on the 3-hydroxy group of bile acids [25,37], no strong discrimination between the two epimers was found in the case of human sulphotransferase. With C₂₀ bile acids, both liver cytosol and purified steroid/bile acid sulphotransferase accepted a 3-hydroxy group with α or β configuration with almost equal efficiency. A number of monohydroxylated short-chain bile acids has been identified in the developing organism and also, but to a lesser extent, in the adult [38–40]. They are known to be secreted as glucuronides [41,42]. Since some short-chain bile acids are metabolically derived from steroid hormones, their analysis may be important to the understanding of hormone turnover and balance. We have demonstrated that all short-chain bile acids are very good substrates for human sulphotransferase. However, the activity of the cytosol fraction as well as that of the purified enzyme was not correlated with the side-chain length of the bile acid in a simple way; the activity was equally high for C₂₄ and C₂₁ compounds, with the remaining compounds being sulphated at a 2–3 times lower rate. The sulphation by human hepatic sulphotransferase of bile acids that carry a hydroxy group in position 6 has not been studied previously. Therefore the relatively high activity of the

Scheme 1. Possible routes of LA detoxification and elimination in human liver

Abbreviation: UDPGT, UDP-glucuronosyltransferase (EC 2.4.1.17).

sulphotransferase toward $3\alpha,6\beta$ -diOH is noteworthy, especially in comparison with its positional isomer, $3\alpha,6\alpha$ -diOH, which was a poor substrate under our conditions. Conjugation of LA with taurine decreases the reaction rate of human hepatic sulphotransferase. Tauroolithocholic acid was sulphated at half of the rate found for LA. This is in contrast with the rat liver bile acid sulphotransferase I, which preferentially accepts amidated LA [9].

A broader question concerns the physiological function of bile acid sulphation in humans. Sulphation constitutes a part of a metabolic response to toxic hydrophobic bile acids, especially to monohydroxylated LA. As mentioned above, the monohydroxylated bile acids, LA and tauroolithocholic acid, were sulphated at rates that were significantly higher than those for all polyhydroxylated bile acids tested. This is in agreement with the numerous reports on the isolation of 3-O-sulphates of LA from urine and bile under normal and pathological conditions [13,14,16,19].

It is tempting to speculate on the significance and relative importance of direct sulphation in comparison with the alternative detoxification pathways, namely hydroxylation and glucuronidation of LA (Scheme 1). The extensive sulphation of LA is in contrast with the marginal rates of glucuronidation of this compound in the human liver [36]. Glucuronidation becomes prominent only after the preceding 6α -hydroxylation of LA and $3\alpha,7\alpha$ -diOH. The 6α -hydroxylated bile acids thus formed are efficiently glucuronidated and excreted [22,43,44], but they do not undergo sulphation (the present paper). Therefore we propose that 3-O-sulphation and 6α -hydroxylation, followed by 6-O-glucuronidation, constitute mutually exclusive routes of detoxification of hydrophobic bile acids. Interesting insights could be obtained from the comparison of the K_m values for these processes. In the present work we have measured the K_m of the DHEA sulphotransferase for LA; the value ($1.5 \mu\text{M}$) is low and may be in the range of LA concentrations present in liver cells. This points to a physiological significance of this particular detoxification pathway. The K_m of the LA 6α -hydroxylase, the rate-limiting step of the alternative pathway [22], is not known. Its determination will allow an estimation of the role of the coupled hydroxylation-glucuronidation reaction in LA excretion.

In summary, it can be concluded that a sulphotransferase with a broad substrate specificity encompassing steroids and bile acids

exists in human liver. This enzyme, in addition to catalysing the sulphation of DHEA and other steroids, catalyses the formation of 3-O-sulphates of monohydroxylated conventional and short-chain bile acids, and several dihydroxylated bile acids. We have no evidence for the formation of bile acid sulphates by any sulphotransferases different from the above DHEA-sulphating enzyme.

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REFERENCES

- Mulder, G. J. (1981) Sulfation of Drugs and Other Compounds, CRC Press, Boca Raton, FL
- Loof, L. & Hjerten, S. (1980) *Biochim. Biophys. Acta* **617**, 192–204
- Chen, L. J. & Segel, I. H. (1985) *Arch. Biochem. Biophys.* **241**, 371–379
- Gugler, R., Rao, G. S. & Breuer, H. (1970) *Biochim. Biophys. Acta* **220**, 69–84
- Loof, L. & Wengle, B. (1979) *Scand. J. Gastroenterol.* **14**, 513–519
- Loof, L. (1981) *Digestion* **21**, 297–303
- Adams, J. B. & McDonald, D. (1981) *Biochim. Biophys. Acta* **664**, 460–468
- Collins, R. H., Lack, L., Harman, K. M. & Killenberg, P. G. (1986) *Hepatology* **6**, 579–586
- Barnes, S., Buchina, E. S., King, R. J., McBurnett, T. & Taylor, K. B. (1989) *J. Lipid Res.* **30**, 529–540
- Barnes, S., Spenney, J. G. (1982) *Biochim. Biophys. Acta* **704**, 353–360
- Hammerman, K. J., Chen, L. J., Fernandez-Corugedo, A. & Earnest, D. L. (1978) *Gastroenterology* **75**, 1021–1025
- Palmer, R. H. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1047–1050
- Stiehl, A. (1974) *Eur. J. Clin. Invest.* **4**, 59–63
- Stiehl, A., Raedsch, R., Rudolph, G., Gundert-Remy, U. & Senn, M. (1985) *Hepatology* **5**, 492–495
- Nitto, H., Obinata, K., Nakatsu, N., Watanabe, T., Nijima, S., Sasaki, H., Arisaka, O., Kato, H., Yabuta, K. & Miyano, T. (1986) *J. Pediatr. Gastroenterol. Nutr.* **5**, 23–29
- Dooley, J. S., Bartholomew, C., Summerfield, J. A. & Billing, B. H. (1984) *Clin. Sci.* **67**, 61–68
- Loof, L. & Wengle, B. (1982) *Scand. J. Gastroenterol.* **17**, 69–76
- Loof, L. & Nyberg, A. (1983) *Upsala J. Med. Sci.* **88**, 1–8
- Stiehl, A., Czygan, P., Frohling, W., Liersch, M. & Kommerell, B. (1977) *Sulphation of Bile Acids*, pp. 129–138, University Park Press, Baltimore
- Falany, C. N., Vazquez, M. E. & Kalb, J. M. (1989) *Biochem. J.* **260**, 641–646
- Radomska-Pyrek, A., Huynh, T., Lester, R. & St. Pyrek, J. (1986) *J. Lipid Res.* **27**, 102–113
- Radomska-Pyrek, A., Zimniak, P., Irshaid, Y. M., Lester, R., Tephly, T. R. & St. Pyrek, J. (1987) *J. Clin. Invest.* **80**, 234–241
- Tserng, K. Y., Hachey, D. L. & Klein, P. D. (1977) *J. Lipid Res.* **18**, 404–407
- Tserng, K. Y. & Klein, P. D. (1979) *Steroids* **33**, 167–182
- Radomska-Pyrek, A., Zimniak, P., Chari, M., Golunski, E., Lester, R. & St. Pyrek, J. (1986) *J. Lipid Res.* **27**, 89–101
- Foldes, A. & Meek, J. L. (1973) *Biochem. Biophys. Acta* **327**, 365–374
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720
- Porter, W. R. & Trager, W. F. (1977) *Biochem. J.* **161**, 293–302
- Cornish-Bowden, A. & Eisenthal, R. (1978) *Biochem. Biophys. Acta* **532**, 268–272
- Duggleby, R. G. (1981) *Anal. Biochem.* **110**, 9–18
- Todhunter, L. A. (1979) *Methods Enzymol.* **63**, 383–411
- Kane, R. E., Chen, L. J., Herbst, J. J. & Thaler, M. M. (1988) *Pediatr. Res.* **24**, 247–253
- Takikawa, H., Stolz, A. & Kaplowitz, N. (1986) *FEBS Lett.* **207**, 193–197
- Singer, S. S. (1978) *Endocrinology (Baltimore)* **103**, 66–73

37. Kirkpatrick, R. B., Falany, C. N. & Tephly, T. R. (1984) *J. Biol. Chem.* **259**, 6176–6180
38. St. Pyrek, J., Sterzycki, R., Lester, R. & Adcock, E. (1982) *Lipids* **17**, 241–249
39. St. Pyrek, J., Lester, R., Adcock, E. W. & Sanghvi, A. T. (1983) *J. Steroid Biochem.* **18**, 341–351
40. St. Pyrek, J., Little, J. M. & Lester, R. (1984) *J. Lipid Res.* **25**, 1324–1329
41. Little, J. M., St. Pyrek, J. & Lester, R. (1983) *J. Clin. Invest.* **71**, 73–80
42. Shattuck, K. E., Radomska-Pyrek, A., Zimniak, P., Adcock, E. W., Lester, R. & St. Pyrek, J. (1986) *Hepatology* **6**, 869–873
43. Marschall, H. U., Matern, H., Egestad, B., Matern, S. & Sjøvall, S. (1987) *Biochim. Biophys. Acta* **921**, 392–397
44. Parquet, M., Pessah, M., Sacquet, E., Salvat, C. & Raizman, A. (1988) *Eur. J. Biochem.* **171**, 329–334

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