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Rat Liver Cytosolic Hydroxysteroid Sulfotransferase (Sulfotransferase a) Catalyzing the Formation of Reactive Sulfate Esters from Carcinogenic Polycyclic Hydroxymethylarenes

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SUMMARY

Female rat liver cytosol contained at least three sulfotransferases (STs) that were separable on a DEAE-Sephadex A-50 column and transformed the carcinogen 5-hydroxymethylchrysene (5-HCR) to the potent mutagen 5-HCR sulfate. The STs also catalyzed sulfation of dehydroepiandrosterone (DHA), a typical substrate for hydroxysteroid STs. Of these three isozymes, the one (STa) with the highest 5-HCR-sulfating activity was isolated and purified (100-fold) as a homogeneous protein, in 15% yield, by successive column chromatography on agarose modified with 3′-phosphoadenosine 5′-phosphate as an affinity ligand and on Sephadex G-100. Purified STa was classified as a hydroxysteroid ST because the 5-HCR- and DHA-sulfating activities were inseparable from each other throughout the purification steps. Sulfation of 5-HCR by purified STa was competitively inhibited by DHA. STa also catalyzed sulfation of other potent carcinogens, 7-hydroxymethylbenz[*a*]anthracene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, and 7,12-dihydroxymethylbenz[*a*]anthracene, to produce sulfate esters with high reactivity and mutagenicity. However, STa had no activity with 4-nitrophenol, a typical substrate for phenol STs, or with *N*-hydroxy-2-acetylaminofluorene. STa had a pI value of 6.4 and existed on a gel filtration column as a homooligomer of a subunit protein with M_r 30,500, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The *N*-terminal amino acid sequence of STa was as follows: Pro-Asp-Tyr-Thr-Trp-Phe-Glu-Gly-Ile-Pro-Phe-Pro-Ala-Phe-Gly-Ile-Pro-Lys-Glu-Thr-. Immunoblot analysis of female and male rat liver cytosol, carried out by using rabbit antiserum raised against the purified enzyme STa and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicated that the female liver contained a much higher level of the enzyme than did the male liver. The marked sex difference in STa level was in good accordance with the previous demonstration that cytosol from the liver of female rats catalyzed sulfation of 5-HCR to a greater extent than did cytosol from the liver of male rats.

The very weak carcinogens chrysene and BA are known to be turned into extremely potent carcinogens by introduction of a methyl group to the 5-position for chrysene and the 7- or/and 12-positions (L region) for BA. For instance, DMBA is the most potent carcinogen among known carcinogenic polycyclic arenes, and the carcinogenicity of 5-methylchrysene is as potent as that of benzo[*a*]pyrene (1–4). Extensive studies have been made with these methylarenes to survey their active metabolites, in relation to the formation of dihydrodiol-epoxides by liver microsomal cytochromes P-450 and epoxide hydrolase in untreated rats and rats treated with polychlorinated biphenyls or 3-methylcholanthrene (2–6).

In the untreated rat liver microsomes, however, oxidation of the strongly carcinogenic methylarenes by the monooxygenases has been demonstrated to take place predominantly at their side chain methyl groups (benzylic carbons), to give the corresponding hydroxymethylarenes (4–7), and all of these metabolites are also potent carcinogens (6, 8, 9).

We have reported the formation of highly reactive sulfate esters, with potent intrinsic mutagenicity toward *Salmonella typhimurium* TA98, from the carcinogens 7-HBA (10), 7-HMBA (11, 12), 12-HMBA (12), DHBA (13), and 5-HCR (14) in rat liver cytosol fortified with PAPS, the cosubstrate for STs. The sulfate esters reacted as electrophiles with the nucleophilic groups of proteins and DNA, through their methylene carbons, with concomitant elimination of sulfate anion. The reactive sulfate esters of 5-HCR (15), 7-HMBA (16), and DHBA (17) bound covalently with high selectivity to the exo-

ABBREVIATIONS: AAF *N*-sulfate, 2-acetylaminofluorene *N*-sulfate; BA, benz[*a*]anthracene; DHA, dehydroepiandrosterone; DHBA, 7,12-dihydroxymethylbenz[*a*]anthracene; DMBA, 7,12-dimethylbenz[*a*]anthracene; 7-HBA, 7-hydroxymethylbenz[*a*]anthracene; 5-HCR, 5-hydroxymethylchrysene; 7-HMBA, 7-hydroxymethyl-12-methylbenz[*a*]anthracene; 12-HMBA, 12-hydroxymethyl-7-methylbenz[*a*]anthracene; HPLC, high pressure liquid chromatography; *N*-OH-AAF; *N*-hydroxy-2-acetylaminofluorene; *N*-OH-MAB, *N*-hydroxy-*N*-methyl-4-aminoazobenzene; 4-NP, 4-nitrophenol; PAP, 3′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; SD, Sprague-Dawley; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ST, sulfotransferase; STa, hydroxysteroid sulfotransferase a; TBA, tetra-*n*-butylammonium.

cyclic amino groups of the purine bases of calf thymus DNA at significant rates, probably with ion pair complex formation of the strongly anionic water-soluble metabolites with the basic nitrogens of the DNA bases as a driving force for their interactions with the DNA molecules. The bifunctional alcohol DHBA was regiospecifically sulfated in rat liver cytosol to give the sole reactive metabolite, DHBA 7-sulfate, as a result of a steric hindrance effect of C_1 in the benzo ring (C_1–C_4) on the 12-hydroxymethyl group (13, 17).

A synthetic sulfate ester of the carcinogen 6-hydroxymethylbenzo[*a*]pyrene has been also demonstrated not only to be an intrinsic mutagen toward TA98 (18) but also to show stronger carcinogenicity to mouse skin than does the parent alcohol (19). The reactive sulfate esters of 7-HMBA (20) and 6-hydroxymethylbenzo[*a*]pyrene (21) have recently been strongly suggested by Surh *et al.* to play important roles in covalent binding of these carcinogenic alcohols to hepatic chromosomal DNA in newborn rats.

The rat liver cytosolic hydroxymethylarene sulfation was shown with 5-HCR (22) and DHBA (17) to be strongly inhibited by DHA sulfate, a typical inhibitor for hydroxysteroid STs (23, 24) that plays an important role in endogenous steroid metabolism, but little inhibited by pentachlorophenol and 2,6-dichloro-4-nitrophenol, both typical phenol ST inhibitors (25, 26). Moreover, the cytosolic 5-HCR sulfation occurred at a much higher rate in female than male animals and was competitively inhibited by DHA sulfate (22). In mature rats, the hepatic hydroxysteroid ST activity is much higher in the female than in the male, whereas the phenol ST activity is in contrast to this (23, 27–31).

As early as 1968, King and Phillips (32) and DeBaun *et al.* (33) independently reported metabolic activation of the hepatocarcinogen *N*-OH-AAF by rat liver STs. Later, the ST that mediates *O*-sulfonation of *N*-OH-AAF was identified as phenol ST IV (34). Other hepatocarcinogens, *N*-OH-MAB (35), 1′-hydroxysafrole (36), and 1′-hydroxy-2′,3′-dehydroestragole (37), were also demonstrated, using the phenol ST inhibitors, to be activated by phenol STs *in vivo*.

The present study was undertaken to obtain direct evidence for the participation of hydroxysteroid STs in the metabolic activation of the carcinogenic hydroxymethylarenes in rat liver. This paper deals with 1) the isolation and purification of an ST, designated as STa, from the female rat liver cytosol, 2) the identification and characterization of STa as a hydroxysteroid ST, and 3) the demonstration of a marked sex difference in hepatic cytosolic level of this enzyme in the rat (female ≫ male), using immunoblot analysis carried out with rabbit antiserum raised against the purified enzyme.

Experimental Procedures

Materials. [1,2,6,7-^{3}H]DNA (97.3 Ci/mmol) and [1,2,6,7-^{3}H]hydrocortisone ([^{3}H]cortisol) (99 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled steroids, 4-NP, TBA bromide, *n*-butanol, L-methionine, AMP (2Na), ADP (2Na), and methylene blue were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). DEAE-Sephadex A-50, Sephadex G-100, agarose-3′,5′-ADP Type 2 (PAP-agarose), and pI marker proteins were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). An anti-rabbit IgG (heavy and light chains specific) and a horseradish peroxidase anti-peroxidase complex preparations were purchased from Organon Teknica Co. (West Chester, PA) and Seikagaku Kogyo Co. (Tokyo, Japan), respectively; 3,3′-diaminobenzidine, bovine serum albumin (fraction V), pig heart lactate dehydrogenase, and molecular weight marker proteins were from Sigma Chemical Co. (St. Louis, MO).

DHBA (7), DHBA-7-sulfate (Na) (13), 7-HBA (38), 7-HBA sulfate (Na) (10), 7-HMBA (7), 7-HMBA sulfate (Na) (11), 5-HCR (39), [^{3}H]5-HCR (22), 5-HCR sulfate (Na) (14), *N*-OH-AAF (40), AAF *N*-sulfate (K) (41), PAPS, and PAP (42) were synthesized as previously reported. 1- and 3-methylmercapto-AAFs were prepared by the direct reaction of AAF *N*-sulfate with L-methionine, as previously reported (43), and were purified by HPLC.

Enzyme assays. Standard assays of the ST activities toward 4-NP and DHA were performed in a final volume of 1 ml of 0.1 M Na_2HPO_4/KH_2PO_4 buffer (pH 6.8) containing the substrate (100 nmol) dissolved in dimethyl sulfoxide (50 μl), PAPS (120 nmol), and an enzyme fraction diluted with the buffer. Enzymatic reactions were started by the addition of the substrate solutions, and the mixtures were incubated for 1 hr at 37°. The sulfate esters formed were extracted as hydrophobic methylene blue complexes with chloroform and were measured by absorbance at 651 nm, as previously reported (44).

For measurement of the ST activity toward 5-HCR, [^{3}H]5-HCR (100 nmol, 0.1 μCi) dissolved in dimethyl sulfoxide (50 μl) was added to a 5-min preincubation mixture (37°) in 0.1 M Na_2HPO_4/KH_2PO_4 buffer (pH 7.4, 950 μl) containing PAPS (120 nmol) and an enzyme fraction diluted with the buffer, and the mixture was incubated for 15 min at 37°. The reaction was terminated by rapid cooling in an ice bath, and the radioactive sulfate ester formed was extracted as a hydrophobic TBA complex with *n*-hexane/ethyl acetate (1:1) together with the substrate from the mixture, transferred as a sodium salt into an aqueous solution of sodium perchlorate and carbonate, and measured by the liquid scintillation counting method previously reported (22).

Enzyme activities of purified STa (2.43 μg of protein) toward 7-HBA, 7-HMBA, DHBA, and *n*-butanol were measured by the determination of PAP formed from PAPS as a result of sulfation of these alcohols incubated for 1 hr under the same conditions as described above for 5-HCR. After the incubation, an aqueous solution (10 μl) of AMP (10–50 nmol), an internal standard, was added to the mixture, and it was extracted three times with an equal volume of a mixture of *n*-hexane and ethyl acetate (5 ml), which was presaturated with the 0.1 M phosphate buffer, to remove the unreacted substrate. The residual aqueous phase was subjected to HPLC carried out on a TSKgel DEAE 5-PW (5 × 50 mm, Tosoh Co., Tokyo, Japan) column. AMP, PAP, and PAPS were eluted at retention times of 2.5, 3.7, and 5.4 min, respectively, from the anion exchange column, at a flow rate of 1 ml/min, with 50 mM Tris·HCl buffer (pH 7.5) containing 0–0.3 M KCl in linear gradient manner. The chromatogram was monitored by absorbance at 260 nm. No detectable PAP formation took place from PAPS either when the substrate was omitted from the incubation mixture or when a boiled enzyme preparation was used.

The sulfating activity for *N*-OH-AAF of purified STa was determined by derivatization of the resulting AAF *N*-sulfate into 1- and 3-methylmercapto-AAFs, as described by DeBaun *et al.* (43), using liver cytosol from male SD rats (weighing 180–200 g) as a positive control. Following extraction with ethyl acetate containing anthrone as an internal reference, the methylmercapto-AAFs were determined by HPLC on Nucleosil $7C_{18}$ (7-μm particle size, 3.9 mm × 30 cm) eluted with methanol/water (7:3, 0.7 ml/min). Under these HPLC conditions, anthrone and 1- and 3-methylmercapto-AAFs were eluted at 23.0, 26.5, and 17.2 min, respectively. Under the above incubation conditions, *N*-OH-AAF was sulfated by the male rat liver cytosol at a rate of 24.4 nmol/mg of protein/20 min.

Blank runs for the enzyme assays were carried out by using boiled cytosol and enzyme preparations, and the assay data were expressed as arithmetic mean values of at least three experiments.

Enzyme purification. All of the following procedures were performed at 4°. Three female SD rats (8 weeks of age; Tokyo Laboratory Animals Science Co. Ltd., Tokyo) were decapitated, and their livers were removed rapidly. The livers (24 g) were homogenized in 2 volumes

of ice-cold 50 mM Tris·HCl buffer (pH 7.5) containing 250 mM sucrose and 3 mM 2-mercaptoethanol (buffer 1), using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 35,000 × *g* for 30 min, and the supernatant was recentrifuged at 105,000 × *g* for 1 hr to obtain a soluble supernatant fraction.

The columns used for separation of enzymes were all previously equilibrated with buffer 1. The liver supernatant fraction (40 ml, 1.138 mg of protein) was applied to a DEAE-Sephadex A-50 column (3.5 × 60 cm), which was eluted at a flow rate of 90 ml/hr with buffer 1 (900 ml) and then with buffer 1 (900 ml) containing 0–0.3 M KCl in linear gradient manner. The column effluent was collected in 12-ml fractions. Fractions 75–90 were combined and directly applied to a PAP-agarose column (1.5 × 10 cm). After the column was washed with buffer 1 (50 ml), it was eluted at a flow rate of 80 ml/hr with buffer 1 containing 0–20 mM ADP in linear gradient manner. The column effluent was collected in 8-ml fractions. Affinity chromatographic fractions 63–103 were combined and concentrated to 12 ml by ultrafiltration (UK-10 membranes; Advantec-Toyo, Tokyo). The concentrated fraction was applied to a Sephadex G-100 column (2 × 100 cm) that was eluted with buffer 1 at 60 ml/hr, with collection of the effluent in 5-ml fractions. Gel filtration chromatographic fractions 15–22 were combined, concentrated to 10 ml by ultrafiltration (UK-10 membrane), and stored at −80°.

Protein concentrations were measured by the method of Lowry *et al.* (45), with bovine serum albumin as a standard.

Electrophoresis. SDS-PAGE was carried out on 15% acrylamide gel plates by the method of Laemmli *et al.* (46).

Isoelectric focusing to obtain the pI value of the enzyme was performed for 1 hr at 5-W constant power, using 7.5% polyacrylamide gel plates containing 3.2% Bio-Lite (pH 3/10; Bio-Rad Laboratories, Richmond, CA). Isoelectric focusing plates were calibrated for pH with pI 5.2–10.25 markers (Pharmacia Fine Chemicals).

Preparation of antiserum. Two male New Zealand white rabbits, weighing 2.4 and 2.5 kg, were used for the preparation of the antiserum raised against purified STa according to the method of Oesch and Bentley (47). Each of the animals received two injections of an emulsion (0.5 ml) of the commercial Freund's complete adjuvant preparation (0.25 ml; Difco Laboratories, Detroit, MI) and the purified enzyme (0.5 mg) in physiological saline, at a 1-week interval, through the foot pad and then an intravenous injection of the enzyme (50 μg) solution in saline (0.5 ml) on day 10 after the second injection. On day 5 after the last injection, the animals were bled for collection of the antiserum.

Absorbance measurements and HPLC. A Hitachi model U-3200 spectrophotometer was used for absorbance measurements of protein concentrations in the column chromatographic fractions. Ion exchange HPLC for the measurement of PAP was carried out on an ATTO model Constametric-II liquid chromatograph equipped with a Jasco model UVIDEC-100-IV spectrophotometer. Gel filtration HPLC was carried out on a TSKgel G2000SW (8 mm × 30 cm; Tosoh) column. Ion pair HPLC for the identification of the sulfate esters formed from hydroxymethylarenes was carried out with the above chromatograph on an octadecylsilica column (Nucleosil $7C_{18}$, 7-μm particle size, 3.9 mm × 30 cm) eluted at a flow rate of 0.8 ml/min with methanol/water (16:9 for DHBA and its 7-sulfate and 3:1 for the others) containing 2 mM TBA bromide. Under the ion pair HPLC conditions used, the sulfate ester-TBA complexes and the corresponding alcohols were eluted at 9.9 and 15.0 min for DHBA 7-sulfate and DHBA (monitored at 295 nm), 6.5 and 15.0 min for 7-HBA sulfate and 7-HBA (291 nm), 6.9 and 17.2 min for 7-HMBA sulfate and 7-HMBA (295 nm), and 10.1 and 21.1 min for 5-HCR sulfate and 5-HCR (269 nm), respectively.

***N*-Terminal amino acid sequence analysis of STa.** Automatic Edman degradation of purified STa, desalted by HPLC as described below, was carried out with an Applied Biosystems model 470A gas phase sequence analyzer and a Spectra Physics SP8700XR phenylthiohydantoin analyzer.

Desalting of the enzyme for *N*-terminal amino acid sequence analysis was carried out on an octadecylsilica column (μ Bondapasphere, 5-μm particle size, 3.9 mm × 15 cm) eluted at a flow rate of 1 ml/min with 0–100% (v/v, 2.5%/min) acetonitrile in 0.1% trifluoroacetic acid, in linear gradient manner (monitored at 214 nm). Under these HPLC conditions, the subunit protein of STa was eluted at 24.4 min as a single peak.

Results

Isolation and purification of STa sulfating 5-HCR from female rat liver cytosol. The 5-HCR-sulfating activity of female rat liver cytosol was eluted as at least three major peaks from a DEAE-Sephadex A-50 column to which the cytosol was directly applied (Fig. 1A). These activity peaks were mostly separated from the major activity peaks for phenol STs, which were monitored by 4-NP. However, the peaks of 5-HCR-sulfating activities were inseparable from those of the activities of hydroxysteroid STs, which were monitored by the endogenous steroid DHA.

Anion exchange column chromatographic fractions 75–90, which had the highest 5-HCR-sulfating or hydroxysteroid ST (STa) activity and little phenol ST activity, were combined and subjected to PAP-agarose affinity column chromatography to collect ST proteins selectively. The 5-HCR- and DHA-sulfating activities were completely retained on the PAP-agarose column and eluted as an inseparable single peak from the column with a linear gradient of ADP, an agent for dissociating STs competitively from the PAP sites of the gel (Fig. 1B). The STa fraction obtained did not contain any phenol ST activity (Fig. 1B).

Affinity column chromatographic fractions 63–103 were combined and rechromatographed on a Sephadex G-100 gel filtration column to remove ADP and a very small amount of protein (M_r 55,000 by SDS-PAGE) as the only contaminant. Under these column chromatographic conditions also, the 5-HCR- and DHA-sulfating activities were eluted as an inseparable single peak, in chromatographic fractions 15–22 (Fig. 1C). Thus, STa catalyzing sulfation of 5-HCR as well as of DHA was obtained as a homogeneous protein (M_r 30,500 by SDS-PAGE) in 15% yield from the female rat liver cytosol, with a purification factor of 100 (Table 1).

Properties of purified STa. The purified enzyme did not have any sulfating activity toward *N*-OH-AAF or 4-NP. STa could be stored in buffer 1 at −80° for more than 3 months without any change in enzyme activity. The enzyme solution lost 20% of its activity at 4° after 2 weeks. STa had a pI value of 6.4 and was suggested by HPLC to exist as an oligomer larger than a tetramer in buffer 1 containing 1 M KCl, used as an eluant (0.1 ml/min), because it was not retained on a TSKgel G2000SW gel filtration column. Under the same HPLC conditions, pig heart lactate dehydrogenase (M_r 145,900), used as an internal reference, was retained on the column and eluted at 32.2 min. The purified enzyme was readily coagulated and precipitated on concentration. In addition, it was very hard to resolubilize the precipitate in aqueous media.

The sequence of the first 20 *N*-terminal amino acid residues of STa was chemically determined to be as follows: Pro-Asp-Tyr-Thr-Trp-Phe-Glu-Gly-Ile-Pro-Phe-Pro-Ala-Phe-Gly-Ile-Pro-Lys-Glu-Thr-. The enzyme showed very broad optimum pH curves for sulfations of 5-HCR and DHA, with a large difference in shape (Fig. 2). The maximum enzyme activity was observed at pH 7.0 for 5-HCR and pH 6.0 for DHA, although

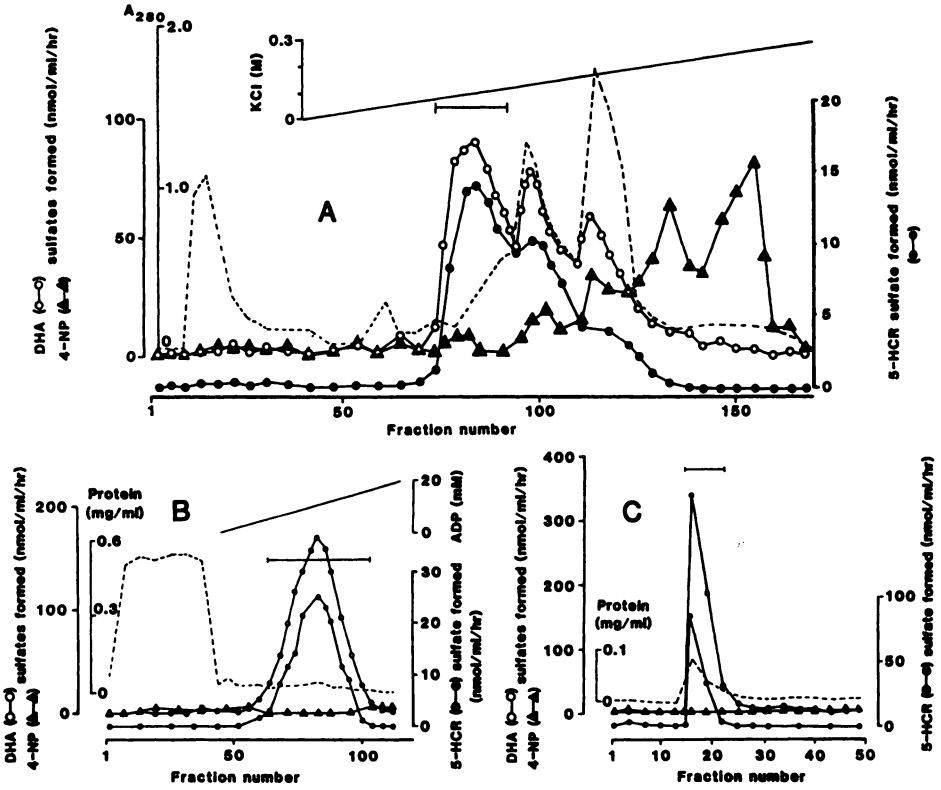

Fig. 1. Elution profiles for hydroxysteroid STs active toward 5-HCR and DHA in chromatographic purification. The enzyme STa was retained on and eluted from the DEAE-Sephadex A-50 anion exchange (A), PAP-agarose affinity (B), and Sephadex G-100 gel filtration (C) columns under the chromatographic conditions described in Experimental Procedures. ST activities were measured with DHA (○), 5-HCR (●), and 4-NP (▲) as sulfate acceptors (see Experimental Procedures). Protein concentrations (– – –) were monitored by absorbance at 280 nm in chromatogram A and by the method of Lowry *et al.* (45) in chromatograms B and C. *Horizontal bars* on the enzyme activity peaks represent the fractions pooled for further chromatographic purification (A and B) and for storage (C).

TABLE 1

Isolation and purification of STa from female SD rat liver cytosol

Step	Specific activities				Purification folds		Yields	
	DHA	5-HCR	4-NP	*N*-OH-AAF	DHA	5-HCR	DHA	5-HCR
	nmol/mg of protein/min						%	
Cytosol	0.58	0.15	0.4		1	1	100	100
DEAE-Sephadex A-50	3.2	1.11	0.1		5.6	7.6	28.1	38.9
PAP-agarose	64.3	16.2	ND[a]		112	110	16.8	16.6
Sephadex G-100[b]	60.3	14.7	ND	ND	105	100	15.0	14.3

[a] ND, not detectable (<0.08 nmol/mg of protein/min).

[b] The enzyme protein lost 10–13% of its activity during condensations by ultrafiltration before and after gel filtration chromatography.

the former showed only a little difference in activity in the range pH 6.0 to 7.5.

As readily assumed from the aforementioned chromatographic data for the enzyme purification, the 5-HCR-sulfating activity of the purified enzyme was competitively inhibited by DHA (Fig. 3). Kinetic parameters obtained for the 5-HCR sulfation by the purified enzyme were K_m of 18.5 μM and V_{max} of 59.9 nmol/mg of protein/min; the inhibition constant K_i was 5.7 μM. However, the double-reciprocal plot for the enzymatic DHA sulfation indicated that higher concentrations of the substrate (≥12.5 μM) inhibited the enzymatic reaction (Fig. 4). Apparent kinetic parameters obtained for the enzymatic sulfation of DHA (≤6.25 μM) were K_m of 2.3 μM and V_{max} of 84.8 nmol/mg of protein/min.

The purified enzyme also catalyzed sulfation of carcinogenic hydroxymethyl-BAs, cortisol, and *n*-butanol (Fig. 5). Specific enzyme activities toward these alcohols were measured by the determination of PAP formed stoichiometrically from the cofactor PAPS during their enzymatic sulfation, because of the extreme instability of the metabolically formed sulfate esters of the hydroxymethyl-BAs 7-HMBA, 7-HBA, and DHBA.

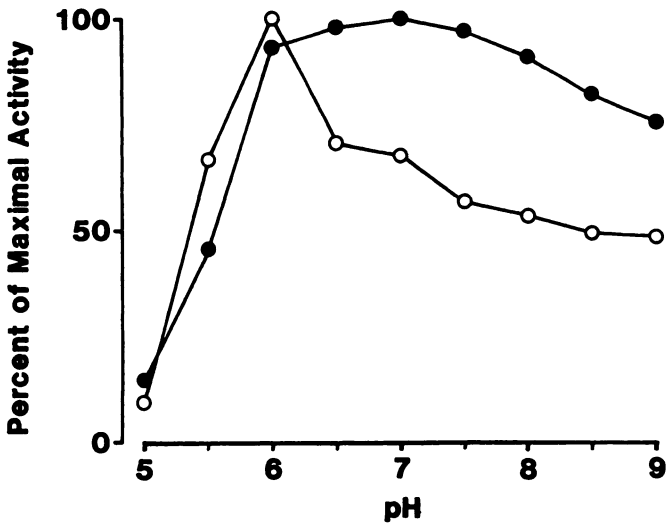

Fig. 2. pH profiles for sulfation of DHA and 5-HCR by purified STa. Enzyme activities toward DHA (○) and 5-HCR (●) were assayed in 0.5 M Tris-acetate buffers.

However, the enzymatic formation of these unstable metabolites was qualitatively confirmed by the extraction with ethyl acetate of the sulfate esters remaining unhydrolyzed in the incubation mixtures, as hydrophobic ion pair complexes with the TBA cation arising from its bromide, and then by their cochromatographic analysis with the corresponding authentic sulfate esters by HPLC. The amounts of metabolically formed PAP in the presence of 5-HCR and DHA were the same as those of their metabolically formed stable sulfate esters, which were directly determined. The three carcinogenic hydroxymethyl-BAs were better substrates than 5-HCR for the purified enzyme. Under the same incubation conditions, cortisol and *n*-

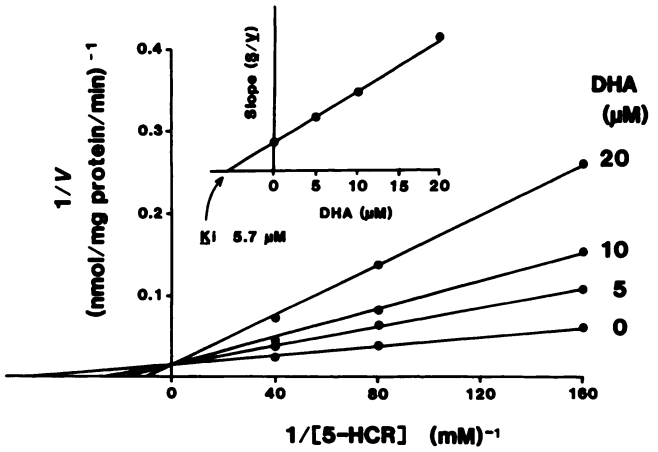

Fig. 3. Competitive inhibition by DHA of 5-HCR sulfation by purified STa. [^{3}H]5-HCR (0.1 μCi, 6.25–25 μM) was used as a varied substrate in the presence and in the absence of DHA. [^{3}H]5-HCR sulfate formed was extracted as a hydrophobic TBA complex from the mixture, separated from the radioactive substrate, and measured by the liquid scintillation counting method described in Experimental Procedures. *Lower*, double-reciprocal plot of the rates of 5-HCR sulfate formation versus concentrations of 5-HCR. *Upper*, plot of concentrations of DHA versus the slopes of the lines in the lower panel.

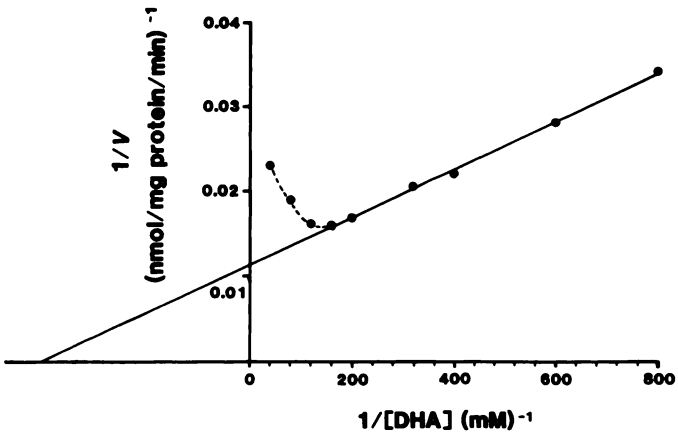

Fig. 4. Double-reciprocal plot of DHA sulfation by purified STa. Assay mixtures contained 0.1 M Na_2HPO_4/KH_2PO_4 (pH 7.4), [^{3}H]DHA (0.1 μCi), 120 μM PAPS, and 2.43 μg of the purified enzyme, in a total volume of 1.0 ml. DHA concentrations were varied as shown. Incubations were carried out for 15 min at 37°. [^{3}H]DHA sulfate formed was directly measured by the liquid scintillation counting method, after complete removal of the radioactive substrate by extractions with *n*-hexane/ethyl acetate (1:1).

butanol were very poor substrates. The hydroxysteroid DHA was the best substrate among the aforementioned alcohols (Fig. 5).

Sex difference in hepatic level of STa. Immunoblot analysis of female and male rat liver cytosol was performed by using a rabbit serum polyclonal antibody raised against purified STa, in order to obtain direct evidence on whether the enzyme played an important role in the previously demonstrated sex difference (female ≫ male) in the sulfate ester formation from 5-HCR in hepatic cytosol (22). After electrophoresis of purified STa and equal amounts of female and male rat liver cytosolic proteins on SDS-polyacrylamide gel, they were transferred to the nitrocellulose membrane for staining of proteins that react with the rabbit anti-STa-IgG, by the peroxidase antiperoxidase method (Fig. 6). The female and male rat liver cytosol both

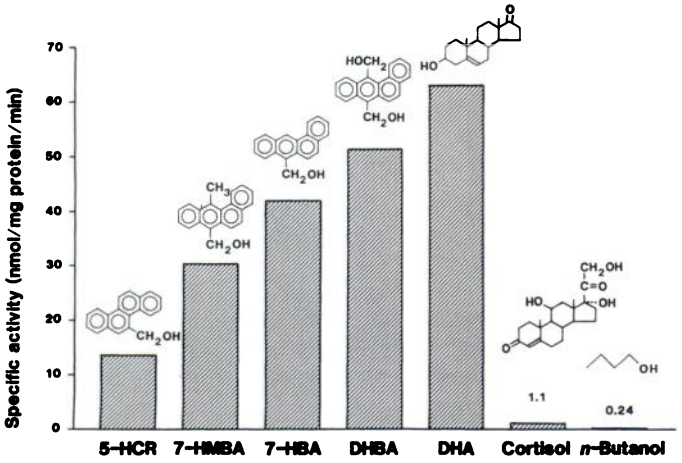

Fig. 5. Substrate specificity of purified STa for carcinogenic hydroxymethylarenes and steroidal and nonsteroidal alcohols. The activities were measured by the determination of enzymatic formation of PAP from PAPS, as described in Experimental Procedures.

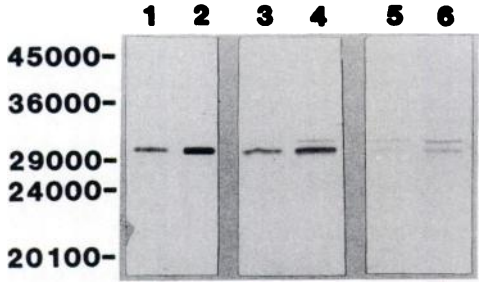

Fig. 6. Immunoblot analysis of the hydroxysteroid ST in female and male rat liver cytosols. Protein samples were resolved by SDS-PAGE (15% gel) and transferred electrophoretically to a nitrocellulose membrane. The membrane was sequentially incubated with 3% bovine serum albumin, rabbit antiserum (at dilution of 1/1,500) raised against the purified hydroxysteroid ST, goat anti-rabbit IgG, rabbit peroxidase antiperoxidase, and finally 50 mM Tris·HCl buffer, pH 7.5, containing hydrogen peroxide and 3,3′-diaminobenzidine, as previously reported (48). *Lanes 1* and *2*, 0.125 and 0.25 μg of purified STa; *lanes 3* and *4*, 5 and 10 μg of protein of female rat liver cytosol; and *lanes 5* and *6*, 5 and 10 μg of protein of male rat liver cytosol, respectively. *Horizontal bars on the left*, molecular weight markers used: ovalbumin (M_r 45,000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (M_r 36,000), bovine erythrocyte carbonic anhydrase (M_r 29,000), bovine pancreas trypsinogen (M_r 24,000), and soybean trypsin inhibitor (M_r 20,000).

contained the M_r 30,500 protein, corresponding to the subunit of STa, and a protein with an M_r value of 32,000. However, there was a marked difference in cytosolic levels of the subunit protein of STa between female and male rat livers (female ≫ male), whereas little difference was observed in the hepatic levels of the other immunoreactive M_r 32,000 protein between the two sexes of the animals.

Discussion

STa, isolated from female SD rat liver cytosol and purified to homogeneity in the present study, catalyzed sulfation not only of a variety of carcinogenic hydroxymethylarenes, including 5-HCR, but also of the endogenous steroids DHA and cortisol, which are typical substrates for all of the known isozymes of rat liver hydroxysteroid ST (27, 28), with a large difference in specific activity (DHA ≫ cortisol). In addition, sulfation of 5-HCR by this enzyme was competitively inhibited by DHA. The purified enzyme had no sulfating activity toward

4-NP, a typical substrate for all of the known isozymes of rat liver phenol ST (27, 28). The phenol STs have been demonstrated to be unable to catalyze sulfation of the endogenous steroids (27, 28). Therefore, STa could be one of the isozymes of hydroxysteroid ST.

The hydroxysteroid ST activity is well known to be higher in liver cytosol from female SD rats than in that from male animals (23, 27–31). Immunoblot analysis of female and male SD rat liver cytosol indicated that the female rat liver cytosol had a much higher level of STa than did the liver cytosol of male animals. This is coincident with our previous demonstration that enzymatic 5-HCR sulfation took place at a much higher rate in liver cytosol from female SD rats than in that from the male animals (22). Thus, the first direct evidence was obtained in the present study for the participation of a hydroxysteroid ST in metabolic activation of carcinogens, whereas phenol STs had been demonstrated or suggested to play an important role in metabolic activation of the carcinogens *N*-OH-AAF (32, 33, 43), *N*-OH-MAB (35), 1′-hydroxysafrole (36), and 1′-hydroxy-2′,3′-dehydroestragole (37).

Hydroxysteroid (or alcohol) STs of female SD rat liver cytosol have been extensively studied by Jakoby and his co-workers (23, 24, 28, 49). They suggested the presence of at least several DHA-sulfating enzymes in the cytosol, which were separable on a DEAE-cellulose column, and they isolated and purified three homooligomeric isozymes, ST1 (pI 5.0 and subunit M_r 28,000) (23), ST2 (pI 7.0 and subunit M_r 32,000) (24), and ST3 (pI 6.1 and subunit M_r 60,000) (28, 49). Singer and his co-workers (27, 30, 31) have also made a great contribution to this research field. The latter group showed the presence of at least three glucocorticoid-sulfating enzymes in female SD rat liver cytosol, STI (pI 6.5 and subunit M_r 28,000) (30), STII (not isolated), and STIII (pI not determined and subunit M_r 30,000) (27, 30, 50). The Singer's STs also catalyze sulfation of DHA (27, 30, 50), and Jakoby's enzymes were also active toward cortisol (23, 24). The two groups of investigators purified the STs in a completely different manner and, hence, no direct information is available on the identity of their isozymes. The aforementioned molecular data on their STs would suggest that at least five hydroxysteroid STs exist in the female SD rat liver cytosol.

The method used for the purification of STa in the present study was very similar to that proposed by Singer (30). However, our purified enzyme (pI 6.4 and subunit M_r 30,500) may differ from his STI in both pI and subunit M_r values and from STIII in the ratio of enzyme activities toward DHA and cortisol (30, 50). They reported that STIII catalyzed the sulfation of cortisol in preference to DHA. In contrast to this, our enzyme was much more active toward DHA than cortisol. Therefore, the identity of our STa with their purified isozymes is equivocal at present.

Very recently, Barnes *et al.* (51) reported the isolation and purification of a bile acid ST (pI 5.3 and subunit M_r 30,000) from female SD rat liver cytosol. The enzyme, named bile acid STI, catalyzed not only sulfation of the 3-hydroxyl groups of a variety of bile acids and their precursors but also sulfation of DHA in preference to cortisol. They also reported the *N*-terminal amino acid sequence of their enzyme, determined by the Edman degradation method. So far as we know, this was the first information on the *N*-terminal amino acid sequence of the ST catalyzing sulfation of hydrophobic substrates, including both steroids and xenobiotics. The bile acid STI, however, differs obviously from our STa, based on the *N*-terminal amino acid sequences. The *N*-terminal amino acid sequence of STa had an extremely strong homology with that of bile acid STI, and they differed from each other by only one amino acid in the sequence from the *N*-terminal to 20th amino acid residue; the 17th amino acid residue was proline for STa and serine for bile acid STI.

Isolation and purification of the other two isozymes, STb and STc, catalyzing sulfation of 5-HCR are now in progress in our laboratory.

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