We report here a systematic investigation on the differential activities of the M-form and P-form PSTs toward a variety of drug compounds. The kinetics of sulfation of the two enantiomeric forms of the chiral drug isoproterenol by the M-form PST was examined. Moreover, the effects of divalent metal cations on the drug-sulfating activities of these two enzymes were studied.

MATERIALS AND METHODS

Materials—Acebutolol, acetaminophen, adenosine 3′-phosphate 5′-phosphosulfate (PAPS), bupivacaine, dexamethasone, dobutamine, lidocaine, (±)isoproterenol, (+)isoproterenol, (−)isoproterenol, 4-methylumbelliferone, (±)metoprolol, minoxidil, DL-propanolol, (±)salbutamol, (±)sotalol, dopamine, *p*-nitrophenol, and adenosine 5′-triphosphate (ATP) were products of Sigma. Carrier-free sodium [^{35}S]sulfate was from ICN Biomedicals. Cellulose thin-layer chromatography (TLC) plates were from EM Science. All other chemicals were of the highest grade commercially available.

Preparation of Purified Human M-Form and P-Form PSTs—Human M-form (SULT1A3) and P-form (SULT1A1) PSTs were cloned, expressed, and purified using the pGEX-2TK Glutathione S-transferase Gene Fusion System based on the procedure previously established (*15*, *16*).

Enzymatic Assay—Sulfotransferase activities of purified M-form and P-form PSTs were assayed using [^{35}S]PAPS as the sulfonate group donor. The standard assay mixture, in a final volume of 25 μl, contained 50 mM potassium phosphate buffer (pH 7.0), 15 μM [^{35}S]PAPS, and 50 μM of the drug compound tested. The enzyme dilutions were prepared in 50 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol and 8 mM dithiothreitol. The reaction was started by the addition of 5 μl of the enzyme preparation, allowed to proceed for 3 min (so that the reaction reached no more than 5–10% of completion) at 37°C, and terminated by heating at 100°C for 2 min. The precipitates were cleared by centrifugation for 1 min, and the supernatant was subjected to the analysis of [^{35}S]sulfated product based on the TLC procedure previously established (*17*). Each experiment was performed in triplicate, together with a control without enzyme. The results obtained were calculated and expressed in nanomoles sulfated product formed/min/mg protein.

Determination of the Stimulatory/Inhibitory Effects of Divalent Metal Cations on the Sulfation of Drug Compounds by Human M-Form or P-Form PST—To determine the patterns of stimulation/inhibition of purified human M-form or P-form PST by divalent metal cations, enzymatic assays were performed in the presence or absence of such cations. Standard assay mixture, in a final volume of 25 μl, contained the test divalent cation (5 mM), 14 μM PAP[^{35}S], 50 mM potassium phosphate buffer (pH 7.0), the enzyme being assayed, and a specified concentration of the drug compound as substrate. Controls containing all the reagents, but without divalent metal cation or with 5 mM EDTA, were assayed in parallel. The reaction was started by the addition of the enzyme and allowed to proceed for 3 min at 37°C, followed by heat inactivation of the enzyme at 100°C for 2 min. The precipitates were cleared by centrifugation for 1 min, and the supernatant was subjected to the analysis of [^{35}S]sulfated product based on the TLC procedure previously established (*17*).

Table 1. **Specific activity of human M-form and P-form PSTs.**[a]

Drug compounds (50 μM)	Specific activity (nmol/min/mg protein)	
	M-PST	P-PST
Acebutolol	N.D.[b]	2.9 ± 0.2
DL-Propanolol	N.D.	2.4 ± 0.1
Bupivacaine	N.D.	2.0 ± 0.1
(±) Metoprolol	N.D.	2.0 ± 0.1
(±) Sotalol	N.D.	2.3 ± 0.1
Dexamethasone	N.D.	2.7 ± 0.1
Dobutamine	68.8 ± 4.4	106.0 ± 2.0
4-Methylumbelliferone	9.4 ± 0.6	95.1 ± 2.9
Lidocaine	0.05 ± 0.02	0.3 ± 0.1
(±)Isoproterenol	147.0 ± 1.1	22.1 ± 1.1
(±)Salbutamol	25.5 ± 0.2	2.9 ± 0.5
Acetaminophen	2.8 ± 0.1	11.7 ± 0.8
Minoxidil	0.5 ± 0.1	4.8 ± 0.1

[a]Data shown represent the mean ± SD derived from three determinations. [b]Activity not detected.

Miscellaneous Methods—[^{35}S]PAPS was synthesized from ATP and carrier-free [^{35}S]sulfate using the bifunctional human ATP sulfurylase/APS kinase and its purity determined as previously described (*18*). The [^{35}S]PAPS synthesized was then adjusted to the required concentration and specific activity by the addition of cold PAPS. SDS–polyacrylamide gel electrophoresis was performed on 12% polyacrylamide gels using the method of Laemmli (*19*). Protein determination was based on the method of Bradford with bovine serum albumin as standard (*20*).

RESULTS AND DISCUSSION

Biological sulfation was first discovered when phenyl sulfate was isolated from the urine of a patient who had been treated with phenol (*21*). This finding had largely positioned the research on sulfation and the responsible sulfotransferase enzymes within the general area of pharmacology/toxicology for over a century. Indeed, a great many studies using experimental animals or volunteer human subjects have demonstrated the metabolism of drugs through sulfation (*22*, *23*). The current study aimed to examine the differential roles of the M-form and P-form PSTs in the sulfation of drug compounds and the regulatory effects of divalent metal cations.

Differential Activities of Human M-Form and P-Form PSTs toward Drug Compounds—Purified M-form and P-form PSTs were assayed using a variety of drug compounds as substrates. Activity data compiled in Table 1 showed that all drug compounds tested (at a concentration of 50 μM in the assay mixture) could be used as substrates by the P-form PST, with dobutamine and 4-methylumbelliferone giving the highest specific activities. In contrast, the M-form PST displayed a more distinct substrate preference, with high activities toward dobutamine, salbutamol, and (±)isoproterenol (all of which are dopamine analogs) and very low or undetectable activities with the other substrates (the concentration of the substrates used was 50 μM). These results showed clearly the differential substrate specificity of the M-form

and P-form PSTs toward the drug compounds tested. It is interesting to note that, in line with its general role as a detoxifying enzyme (*2*, *3*), the P-form PST displays a broader substrate specificity than the M-form PST, which is believed to play a more specific role in the homeostasis of dopamine in the brain and perhaps the detoxification of this and other deleterious monoamines and dopamine analogs in the upper gastrointestinal tract (*8*). Table 2 shows the kinetic constants determined for the sulfation of acetaminophen (one of the most extensively used drugs, which is believed to be detoxified in the liver) and minoxidil (which is activated by conversion to its sulfate-conjugated form) by the P-form PST and the sulfation of dobutamine by the M-form PST. Based on these data, the catalytic efficiency of the M-form PST for the sulfation of dobutamine appeared to be considerably higher than that of the P-form PST for the sulfation of acetaminophen or minoxidil. Previous studies using liver homogenates demonstrated the stereoselective sulfation of (±)isoproterenol (*13*). In view of our previous finding that the M-form PST displayed stereoselectivity for the D-enantiomers of Dopa and tyrosine (*11*), we decided to test the kinetics of sulfation of the two enantiomeric forms of isoproterenol by this enzyme. Results shown in Fig. 1 indicated that the apparent K_m values determined for the two enantiomers of isoproterenol are nearly identical, whereas the V_{max} for the (+)enantiomer is almost two times that for the (−)enantiomer. The preferential sulfation of one enantiomeric form over the other may have important pharmacological implications for drugs that are administered in the racemic form.

Effects of Divalent Metal Cations on the Sulfation of Drug Compounds by Human M-Form and P-Form PSTs—Divalent metal cations are known to play important roles in the function of biological molecules (*24*). Some of them, including manganese, zinc, copper, iron, and cobalt, are essential components of different enzymes, while others such as magnesium and calcium are required for the actions of certain enzymes or proteins. In contrast to these biologically useful metal cations, some metal cations (*e.g.*, lead and mercury) that enter the body primarily as environmental contaminants have been shown to exert deleterious effects (*25*, *26*). Our previous studies have revealed that some divalent metal cations may exert stimulatory or inhibitory effects on the cytosolic STs (*10*, *11*). We were interested in investigating the effects of these divalent metal cations on the sulfation of drug compounds. Enzymatic assays, using

Table 2. **Kinetic constants of human M-form and P-form PSTs with dobutamine, acetaminophen, and minoxidil as substrates.**[a]

	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m
M-form PST			
Dobutamine	14.8	384.6	26.0
P-form PST			
Acetaminophen	430.4	22.1	0.05
Minoxidil	200.6	6.2	0.03

[a]Data shown represent mean ± SD derived from three determinations.

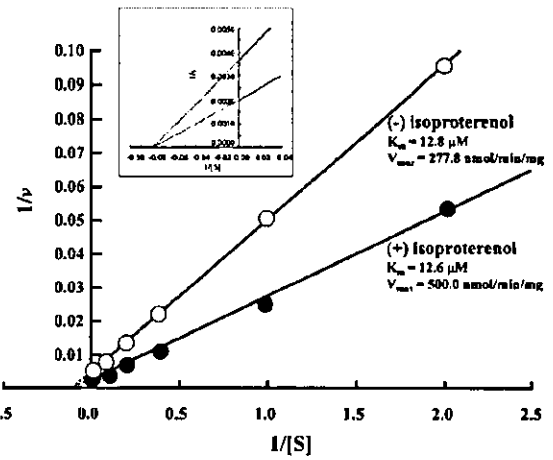

Fig. 1. **Lineweaver-Burk double-reciprocal plot of the human M-form PST with (+)Isoproterenol or (−)Isoproterenol as substrate.** Concentrations of (+)isoproterenol or (−)isoproterenol are expressed in μM, and velocities are expressed in nmol of product formed/min/mg enzyme. Each data point represents the mean value derived from three determinations.

Table 3. **Inhibitory/stimulatory effects of divalent metal cations on the human M-form and P-form PSTs.**[a]

	M-form PST		P-form PST	
	Dobutamine (5.0 μM)	(±) Isoproterenol (10 μM)	Acetaminophen (500 μM)	Minoxidil (250 μM)
	(nmol/min/mg protein)			
Control	112 ± 3	126 ± 1	9.5 ± 0.5	3.1 ± 0.3
$FeCl_2$	73.9 ± 1.1	90.7 ± 2.1	13.1 ± 0.3	3.3 ± 0.2
$HgCl_2$	3.8 ± 0.2	ND[b]	1.4 ± 0.1	0.06 ± 0.02
$CoCl_2$	103 ± 0.2	119 ± 2	5.3 ± 0.2	1.2 ± 0.1
$ZnCl_2$	99.6 ± 0.4	65.5 ± 0.4	3.6 ± 0.1	0.6 ± 0.1
$Pb(CH_3COO)_2$	107 ± 3	120 ± 1	10.5 ± 0.9	3.0 ± 0.1
$CdCl_2$	111 ± 2	122 ± 2	8.7 ± 0.1	2.0 ± 0.2
$MnCl_2$	99.5 ± 1.3	124 ± 3	12.6 ± 0.1	3.7 ± 0.2
$CaCl_2$	114 ± 3	115 ± 3	10.3 ± 0.6	2.8 ± 0.1
$MgCl_2$	113 ± 1	135 ± 2	10.8 ± 0.8	3.5 ± 0.2
$CuCl_2$	0.8 ± 0.1	2.4 ± 1.2	0.05 ± 0.02	0.04 ± 0.02
NaCl	112 ± 3	118 ± 0.3	9.5 ± 0.8	3.0 ± 0.2

[a]Data shown represent mean ± SD derived from three determinations. [b]Activity not detected.

acetaminophen and minoxidil (for the P-form PST) or dobutamine and (±)isoproterenol (for the M-form PST) as substrates (at the concentrations specified), were carried out in the absence or presence of various divalent metal cations at a concentration of 5 mM. As a control for the counter ion, Cl^-, parallel assays in the presence 10 mM NaCl were also performed. Results obtained are compiled in Table 3. The degrees of inhibition or stimulation were evaluated by comparing the activities determined in the presence of metal cations with the activities determined in the absence of metal cations. Both the M-form and P-form PSTs were found to be partially or completely inhibited or stimulated by the majority of the divalent metal cations tested, and both were less sensitive to the effects of physiologically more abundant metal cations such as Mg^{2+} and Ca^{2+}. In contrast, they were more sensitive to the detrimental effects of metal cations that may enter the body as environmental contaminants. At a concentration of 5 mM, Cu^{2+} and Hg^{2+} exerted nearly complete inhibition of the activity of the M-form and P-form PSTs toward the four drug compounds tested. These dramatic inhibitory effects imply that the presence of these metal cations may interfere with the metabolism of drugs through sulfation. To what extent the inhibitory effects of these metal cations on the M-form and P-form PSTs may help prolong the presence of drug compounds *in vivo*, however, remains to be clarified.

Finally, it should be pointed out that, in addition to the differential substrate specificity of the human M-form and P-form PSTs toward drug compounds, recent studies have revealed the genetic polymorphism of the P-form PST (*27*, *28*). It may be important, therefore, to examine the drug-sulfating phenotypes of different P-form PST allozymes. Information of this kind will likely help in understanding the individual differences in drug metabolism and may in the future aid in developing tailored drug regimens that suit individual needs.

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Sulfation of hydroxychlorobiphenyls

Molecular cloning, expression, and functional characterization of zebrafish SULT1 sulfotransferases

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As a first step toward developing a zebrafish model for investigating the role of sulfation in counteracting environmental estrogenic chemicals, we have embarked on the identification and characterization of cytosolic sulfotransferases (STs) in zebrafish. By searching the zebrafish expressed sequence tag database, we have identified two cDNA clones encoding putative cytosolic STs. These two zebrafish ST cDNAs were isolated and subjected to nucleotide sequencing. Sequence data revealed that the two zebrafish STs are highly homologous, being ≈ 82% identical in their amino acid sequences. Both of them display ≈ 50% amino acid sequence identity to human SULT1A1, rat SULT1A1, and mouse SULT1C1 ST. These two zebrafish STs therefore appear to belong to the SULT1 cytosolic ST gene family. Recombinant zebrafish STs (designated SULT1 STs 1 and 2), expressed using the pGEX-2TK prokaryotic expression system and purified from transformed *Escherichia coli* cells, migrated as ≈ 35 kDa proteins on SDS/PAGE. Purified zebrafish SULT1 STs 1 and 2 displayed differential sulfating activities toward a number of endogenous compounds and xenobiotics including hydroxychlorobiphenyls. Kinetic constants of the two enzymes toward two representative hydroxychlorobiphenyls, 3-chloro-4-biphenylol and 3,3′,5,5′-tetrachloro-4,4′-biphenyldiol, and 3,3′,5-triiodo-L-thyronine were determined. A thermostability experiment revealed the two enzymes to be relatively stable over the range 20–43 °C. Among 10 different divalent metal cations tested, Co^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} exhibited considerable inhibitory effects, while Hg^{2+} and Cu^{2+} rendered both enzymes virtually inactive.

Keywords: hydroxychlorobiphenyls; sulfation: sulfotransferase; SULT1: zebrafish.

In mammals (and possibly in other vertebrates), sulfation is known to be a major pathway for the detoxification of xenobiotics as well as the biotransformation of endogenous compounds such as steroid and thyroid hormones, catecholamines, and bile acids [1–3]. The enzymes responsible, called the cytosolic sulfotransferases (STs), catalyze the transfer of a sulfonyl group from the 'active sulfate', 3′-phosphoadenosine-5′-phosphosulfate (PAPS), to a variety of compounds containing hydroxyl or amino groups [4]. Sulfation of these compounds may result in their inactivation/activation or increase their water solubility, thereby facilitating their removal from the body [5,6].

In recent years there have been a number of reports of estrogens and estrogen-like chemicals such as polychlorobiphenyls in the environment having an adverse impact on humans as well as wildlife including reptiles and birds [7,8]. These compounds, collectively referred to as environmental estrogens, are becoming ubiquitous in the environment and are increasingly making their way into the food chain. Considering that sulfation is widely used *in vivo* for the inactivation and/or excretion of xenobiotic compounds, we became interested in the role of this phase II detoxification pathway in the metabolism of environmental estrogens. Our recent studies have demonstrated that some human cytosolic STs, in particular the simple phenol (P)-form phenol ST, are capable of catalyzing the sulfation of several representative environmental estrogens [9,10]. We wanted to investigate further whether wildlife, in particular aquatic animals, are also equipped with ST enzymes that are able to counteract environmental estrogens.

Zebrafish has in recent years emerged as a popular animal model for a wide range of studies [11,12]. Its advantages, compared with mouse, rat, or other vertebrate animal models, include the small size, availability of relatively large number of eggs, rapid development externally of virtually transparent embryo, short generation time, etc. These unique characteristics of the zebrafish make it an excellent model for a systematic investigation on the ontogeny of the expression of individual cytosolic STs and their tissue- and cell type-specific distribution, as well as the physiological

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Abbreviations: ST, sulfotransferase; PAPS, 3′-phosphoadenosine 5′ phosphosulfate; T_3, 3,3′,5-triiodo-L-thyronine; T_4, thyroxine; estrone, 1,3,5[10]-estratrinen-3-ol-17-one; dopa, 3,4-dihydroxyphenylalanine; PST, phenol sulfotransferase.

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relevance of individual cytosolic STs. A prerequisite for using zebrafish in these studies, however, is the identification of the various cytosolic STs and their biochemical characterization.

We report in this communication the molecular cloning and expression of two distinct zebrafish cytosolic STs. The enzymatic activities of purified recombinant enzymes toward a variety of endogenous and xenobiotic compounds including hydroxychlorobiphenyls were tested. Moreover, using a zebrafish liver cell line as a model, the metabolism of environmental estrogens through sulfation was investigated.

Experimental procedures

Materials

p-Nitrophenol, dopamine, L-3,4-dihydroxyphenylalanine (L-dopa), D-dopa, 2-naphthol, 2-naphthylamine, aprotinin, thrombin, bovine insulin, 3,3',5-triiodo-L-thyronine (T_3; sodium salt), thyroxine (T_4), estrone (1,3,5[10]-estratrinen-3-ol-17-one), dehydroepiandrosterone, ATP, SDS, sodium selenite, Hepes, Taps, Trizma base, dithiothreitol, and isopropyl thio-β-D-galactoside were from Sigma Chemical Co. 3-Chloro-4-biphenylol and 4,4'-dihydroxy-3,3',5,5'-tetrachlorobiphenyl were from Ultra Scientific. Two zebrafish cDNA clones, ID 3719883 (GenBank accession number AI588236) and ID 2641807 (GenBank accession number AW422150), encoding cytosolic STs were obtained from Genome Systems, Inc. Ampli*Taq* DNA polymerase was from Perkin Elmer. Takara *ExTaq* DNA polymerase was from PanVera Corporation (Madison, WI, USA). T_4 DNA ligase and all restriction endonucleases were from New England Biolabs. XL1-Blue MRF' and BL21 *Escherichia coli* host strains were from Stratagene. Oligonucleotide primers were synthesized by MWG Biotech. pBR322 DNA/*Mva*I size markers were from MBI Fermentas. pGEX-2TK glutathione S-transferase (GST) gene fusion vector and glutathione Sepharose 4B were from Amersham Biosciences. Recombinant human bifunctional ATP sulfurylase/adenosine 5'-phosphosulfate kinase was prepared as described previously [13]. Ham's F-12 nutrient mixture, Leibivitz's L-15 medium, Dulbecco's modified Eagle's medium, minimum essential medium, and fetal bovine serum were from Life Technologies. Trout serum was from East Coast Biologics, Inc. Zebrafish liver cells were prepared and maintained under conditions established previously [14]. TRI Reagent was from Molecular Research Center, Inc. Total RNAs from whole zebrafish and zebrafish liver cells were prepared using the TRI Reagent according to manufacturer's instructions. Rabbit antiserum against purified recombinant zebrafish SULT1 ST1 was prepared based on the procedure described previously [15]. Renaissance Western Blot Chemiluminescence Reagent Plus was from NEN Life Science Products. Cellulose TLC plates were products of EM Science. Carrier-free sodium [^{35}S]sulfate was from ICN Biomedicals. All other reagents were of the highest grades commercially available.

Molecular cloning of zebrafish cytosolic STs

By searching the expressed sequence tag database, two zebrafish cDNA clones (GenBank accession number AI588236 and AW422150) encoding putative cytosolic STs were identified. These two zebrafish ST cDNAs were purified and subjected to nucleotide sequencing based on the cycle sequencing method using, respectively, M13 forward/M13 reverse and pME18S-5'/pME18S-3' as primers. The nucleotide sequences, as well as the deduced amino acid sequences, of the two cDNAs were analyzed using BLAST search for sequence homology to known cytosolic STs.

Bacterial expression and purification of recombinant zebrafish cytosolic STs

To amplify the two zebrafish ST cDNAs for subcloning into the prokaryotic expression vector pGEX-2TK, two sets of sense and antisense oligonucleotide primers (see Table 1), based on 5'- and 3'- coding regions of the two zebrafish ST cDNAs, were synthesized with *Bam*HI restriction site incorporated at the ends. With each of the two sets of oligonucleotides as primers, PCR in a 100-μL reaction mixture was carried out using *ExTaq* DNA polymerase and pSPORT1 (or pME18S-FL3) harboring the specific zebrafish ST cDNA as template. Amplification conditions were 25 cycles of 45 s at 94 °C, 45 s at 59 °C, and 1 min at 72 °C. The final reaction mixture was applied onto a 1.2% agarose gel and separated by electrophoresis. The discrete PCR product band, visualized by ethidium bromide staining, was excised from the gel and the DNA fragment therein was isolated by spin filtration. After *Bam*HI digestion, the PCR product was subcloned into the *Bam*HI site of pGEX-2TK and transformed into *E. coli* BL21. To verify its authenticity, the cDNA insert was subjected to nucleotide sequencing [16].

Competent *E. coli* BL21 cells, transformed with pGEX-2TK harboring the zebrafish ST cDNA, were grown to $D_{600} \approx 0.5$ in 1 L Luria–Bertani medium supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin, and induced with 0.1 mM

Table 1. Oligonucleotide primers used for PCR amplifications for full-length ZF SULT1 ST1 and ST2 sequences. Recognition sites of the restriction endonuclease in the oligonucleotides are underlined. Initiation and termination codons for translation are in bold.

Sequence	Primer
ZF SULT1 ST1	
Sense	5'-CGCGGATCCATGGACATGCCTGACTTTTCT-3'
Antisense	5'-CGCGGATCCTTAAATCTCAGTGCGGAACTT-3'
ZF SULT ST2	
Sense	5'-CGCGGATCCATGAAACTGGATAGCCGGCCT-3'
Antisense	5'-CGCGGATCCTCATCTTTTGTTTGTAGTCCT-3'

isopropyl thio-β-D-galactoside. After an overnight induction at room temperature, the cells were collected by centrifugation and homogenized in 20 mL ice-cold lysis buffer (20 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) using an Aminco French Press. Twenty μL of 10 mg·mL^{-1} aprotinin (a protease inhibitor) was added to the crude homogenate which was then subjected to centrifugation at 10 000 *g* for 30 min at 4 °C. The supernatant was fractionated using 0.5 mL glutathione Sepharose, and the bound GST fusion protein was treated with 2 mL of a thrombin digestion buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$) containing 5 U·mL^{-1} bovine thrombin. Following a 30-min incubation at room temperature with constant agitation, the preparation was subjected to centrifugation. The recombinant zebrafish ST present in the supernatant collected was analyzed with respect to its enzymatic properties.

Enzymatic assay

The ST activities were assayed using [^{35}S]PAP as the sulfate donor. The standard assay mixture, with a final volume of 25 μL, contained 50 mM potassium phosphate (pH 7.0), 14 μM [^{35}S]PAP (15 Ci·mmol^{-1}), and 50 μM substrate. The reaction was started by the addition of the enzyme (0.25 μg per 25 μL reaction mixture) and allowed to proceed for 3 min at 28 °C. (Amount of enzyme and reaction time were chosen to ensure that there was no more than 5% reaction: the reaction was linear with time and amount of enzyme.) The reaction was terminated by heating at 100 °C for 2 min. The precipitates formed were cleared by centrifugation, and the supernatant was subjected to the analysis of [^{35}S]-sulfated product using the TLC procedure developed previously [17], with butan-1-ol/isopropanol/88% formic acid/water (2 : 1 : 1 : 2; v/v/v/v) as solvent. To examine the pH dependence, different buffers (50 mM sodium succinate at 3.5, 3.75, 4.0 or 4.25; sodium acetate at 4.5, 4.75, 5.0 or 5.25; Mes at 5.5 or 6.0; Mops at 6.5 or 7.0; Taps at 7.5, 8.0, 8.5 or 9.0; Ches at 9.0 or 9.5; and Caps at 9.5, 10.0, 10.5, or 11.0) instead of 50 mM potassium phosphate buffer (pH 7.0) were used in the reactions. For kinetic studies of the sulfation of hydroxychlorobiphenyls, varying concentrations of these latter substrate compounds and 50 mM Mops at pH 7.0 were used. To evaluate their thermostability, the zebrafish STs were first incubated for 15 min at, respectively, 20, 28, 37, 43 and 48 °C, and then assayed for their activities at 28 °C. To determine the stimulatory/inhibitory effects of divalent metal cations, enzymatic assays in the presence or absence of divalent metal cations were performed under standard conditions as described above.

Western blot analysis

To examine the expression of the zebrafish SULT1 ST1, our previously established Western blotting procedure [15] was used with rabbit anti-(zebrafish ST) serum as the probe. Briefly, crude homogenates of zebrafish whole body or cultured zebrafish liver cells, solubilized in SDS sample buffer and heated for 3 min at 100 °C, were separated by SDS/PAGE and electrotransferred onto an Immobilon-P membrane [18]. The blotted membrane was blocked with 5% nonfat dried milk in NaCl/P_i for 1 h and probed with 20 μL rabbit anti-(zebrafish ST) serum. After a 1-h incubation, the membrane was washed with NaCl/P_i, treated with horseradish peroxidase-conjugated secondary antibody in NaCl/P_i containing 5% nonfat dried milk, and processed using the Renaissance Western Blot Chemiluminescence Reagent Plus according to the manufacturer's instructions. Autoradiography was then performed on the processed membrane.

Metabolic labeling of zebrafish liver cells with [^{35}S]sulfate in the presence of environmental estrogens

Zebrafish liver cells were routinely grown in LDF culture medium (50% Leibovitz's L-15, 35% Dulbecco's modified Eagle's medium, 15% Ham's F-12, 10^{-8} M sodium selenite) supplemented with 5% fetal bovine serum, 0.5% trout serum, 0.1 mg·mL^{-1} bovine insulin, 50 μg·mL^{-1} streptomycin sulfate, and 30 μg·mL^{-1} penicillin G. Confluent zebrafish liver cells grown in individual wells of a 24-well culture plate, preincubated in sulfate-free (prepared by omitting streptomycin sulfate and replacing magnesium sulfate with magnesium chloride) minimum essential medium for 4 h, were labeled with 0.2 mL aliquots of the same medium containing [^{35}S]sulfate (0.25 mCi·mL^{-1}), and 100 μM 3-chloro-4-biphenylol or 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl. At the end of a 12-h labeling period, media were collected, spin-filtered, and the [^{35}S]-sulfated 3-chloro-4-biphenylol or 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl were analyzed by TLC.

Miscellaneous methods

[^{35}S]PAPS was synthesized from ATP and carrier-free [^{35}S]sulfate using the bifunctional human ATP sulfurylase/APS kinase and its purity was determined as described previously [19]. The [^{35}S]PAPS synthesized was then adjusted to the required concentration and specific activity by the addition of cold PAPS. SDS/PAGE was performed on 12% polyacrylamide gels using the method of Laemmli [20]. Protein determination was based on the method of Bradford [21] with BSA as standard.

Results and discussion

Although considerable progress has been made in recent years on the cytosolic STs, several fundamental questions concerning their ontogeny, regulation, and physiological involvement still remain to be fully elucidated. The present study was prompted by an attempt to develop a zebrafish model in order to address these important issues. As a first step toward achieving this goal, we have started investigating the various cytosolic STs that are present in zebrafish.

Molecular cloning of the two novel zebrafish cytosolic STs

By searching the zebrafish expressed sequence tag database, we have spotted two cDNA clones encoding putative zebrafish STs. Analysis of the partial nucleotide sequences available for these two cDNA clones via BLAST search confirmed their identity as ST cDNAs (data not shown).

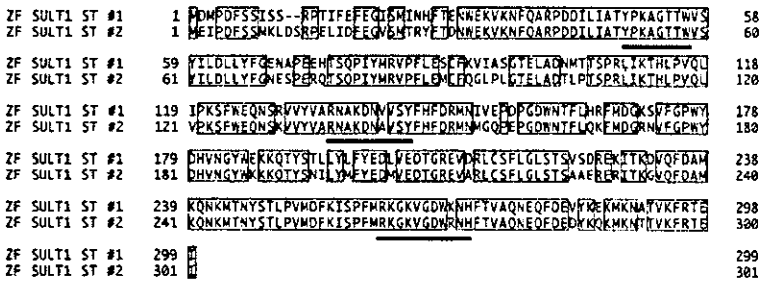

Fig. 1. Amino acid sequence comparison of zebrafish SULT1 ST1 and SULT1 ST2. Residues conserved between the two STs are boxed. Two 'signature sequences' located in the N-terminal and C-terminal regions, and a conserved sequence in the middle region, are underlined.

They were then isolated and subjected to complete nucleotide sequencing in both directions. The nucleotide sequences obtained were submitted to the GenBank database under the accession numbers AY181064 (clone ID 3719883) and AY181065 (clone ID 2641807). Fig. 1 shows the aligned deduced amino acid sequences of these two zebrafish STs. It is noted that the two zebrafish cytosolic STs appeared to be highly homologous, being ≈82% identical in their amino acid sequences. Similar to other cytosolic STs, both zebrafish STs contain the so-called 'signature sequences' (YPKSGTxW in the N-terminal region and RKGxxGDWKNxFT in the C-terminal region; underlined) characteristic of ST enzymes [22]. Of these two sequences, YPKSGTxW has been demonstrated by X-ray crystallography to be responsible for binding to the 5′-phosphosulfate group of PAPS, a cosubstrate for ST-catalyzed sulfation reactions [4], and thus designated the '5′-phosphosulfate binding (5′-PSB) motif' [23]. Both zebrafish STs also contain the '3′-phosphate binding (3′-PB) motif' (residues 135–143 for SULT1 ST1 and residues 137–145 for SULT1 ST2; underlined) responsible for the binding to the 3′-phosphate group of PAPS [23].

Based on the amino acid sequences of known mammalian cytosolic STs, several gene families have been categorized within the cytosolic ST gene superfamily. Two major gene families among them are the phenol ST (PST) family (designated SULT1) and hydroxysteroid ST family (designated SULT2) [22]. The PST family consists of at least four subfamilies, PSTs (SULT1A), Dopa/tyrosine (or thyroid hormone) STs (SULT1B), hydroxyarylamine (or acetylaminofluorene) STs (SULT1C), and estrogen STs (SULT1E). The hydroxysteroid ST family presently comprises two subfamilies, dehydroepiandrosterone STs (SULT2A) and cholesterol STs (SULT2B). Sequence analysis based on BLAST search revealed that the deduced amino acid sequence of zebrafish SULT1 ST1 displayed, respectively, 50%, 50%, and 49% identity to those of mouse SULT1C1, rat SULT1A1, and human SULT1A1 STs [22]. The deduced amino acid sequence of zebrafish SULT1 ST2 displayed, respectively, 51%, 51% and 47% identity to those of human SULT1A1, rat SULT1A1, and mouse SULT1C1 STs [22]. It is generally accepted that members of the same ST gene family share at least 45% amino acid sequence identity, whereas members of subfamilies further divided in each ST gene family are > 60% identical in amino acid sequence [22]. Based on these criteria, the two zebrafish STs, while clearly belonging to the SULT1 gene family, cannot be classified into any of the existing subfamilies within SULT1 (cf. the dendrogram shown in Fig. 2).

Bacterial expression, purification, and characterization of recombinant zebrafish cytosolic STs

The coding sequences of the two zebrafish SULT1 STs were individually subcloned into pGEX-2TK, a prokaryotic expression vector, for the expression of recombinant enzymes in *E. coli*. As shown in Fig. 3, the two recombinant zebrafish SULT1 STs, cleaved from their respective glutathione Sepharose-fractionated fusion proteins, migrated at ≈35 kDa on SDS/PAGE. The purified recombinant zebrafish SULT1 STs were subjected to functional characterization with respect to their enzymatic activities. A pilot experiment showed that both enzymes exhibited strong

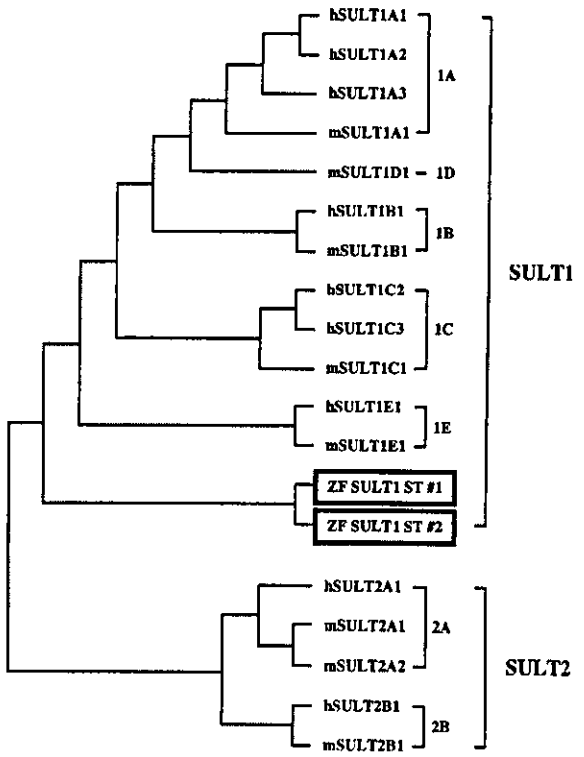

Fig. 2. Classification of zebrafish SULT1 ST1 and SULT1 ST2 on the basis of their deduced amino acid sequences. The dendrogram shows the degree of amino acid sequence homology among cytosolic STs. For references for individual STs, see the review by Weinshilboum *et al.* [22]. h, Human; m, mouse.

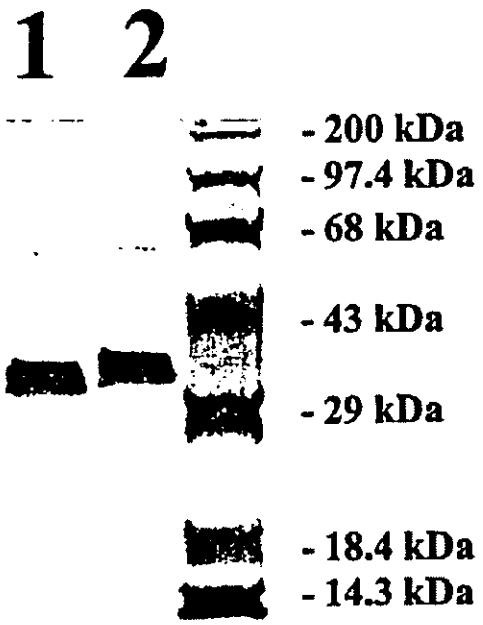

Fig. 3. SDS/PAGE of purified recombinant zebrafish STs. Purified zebrafish SULT1 ST1 (lane 1) and SULT1 ST2 (lane 2) were subjected to SDS/PAGE on a 12% gel, followed by Coomassie blue staining. Protein molecular mass markers: lysozyme (M_r = 14 300), β-lactoglobulin (M_r = 18 400), carbonic anhydrase (M_r = 29 000), ovalbumin (M_r = 43 000), BSA (M_r = 68 000), phosphorylase b (M_r = 97 400), myosin (H-chain; M_r = 200 000).

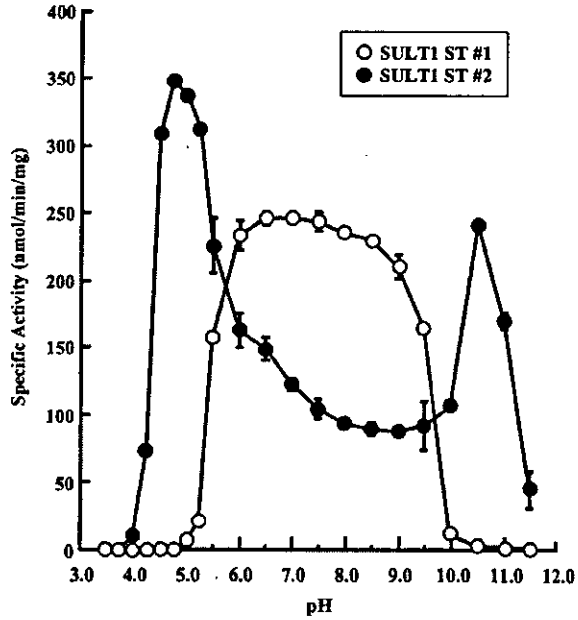

Fig. 4. pH-dependency of the 2-naphthol-sulfating activity of purified zebrafish SULT1 STs1 and 2. The enzymatic assays were carried out under standard assay conditions as described using different buffer systems as indicated. The data represent calculated mean values derived from three experiments.

activities toward 2-naphthol, a typical substrate for PST (SULT1A) enzymes [1–3]. A pH dependence experiment subsequently performed revealed that the zebrafish SULT1 ST1 exhibited a broad pH optimum of pH 6.0–9, while the ZF SULT1 ST2 showed, intriguingly, two optima at pH 4.75 and 10.5 (Fig. 4). Whether the two pH optima of the ZF SULT1 ST2 correspond to two distinct conformational states of the enzyme remains to be clarified. A number of endogenous and xenobiotic compounds were then tested as substrates for the two enzymes. Activity data compiled in Table 2 revealed that, despite their high degree of sequence homology, the two zebrafish STs displayed differential activities toward the various endogenous and xenobiotic compounds tested. Among the endogenous substrates, zebrafish SULT1 ST1 appeared to be more active toward dopamine and T_3, whereas zebrafish SULT1 ST2 was more active toward the thyroid hormones (T_3 and T_4), estrone, and dopa. Whether these activities reflect truly the physiological functions of the two enzymes in zebrafish remains to be clarified. Elucidation of the tissue- or cell type-specific expression of these two enzymes may provide clues in this regard. The two zebrafish STs also exhibited differential activities toward the xenobiotic compounds tested. It is particularly interesting to note that both of them can catalyze the sulfation of the two hydroxychlorobiphenyls tested, with SULT1 ST1 being more effective than SULT1 ST2. Table 3 shows the kinetic constants determined for the two enzymes using 3-chloro-4-biphenylol, 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl or T_3 as substrate. Compared with SULT1 ST2, SULT1 ST1 showed greater K_m and yet

Table 2. Specific activity (nmol substrate sulfated per min·per mg purified enzyme) of zebrafish SULT1 STs 1 and 2 toward endogenous and xenobiotic compounds. Data represent mean ± SD from three experiments. ND, activity not detected.

	SULT1 ST 1	SULT1 ST 2
3,3′,5-Triiodo-L-thyronine	7.9 ± 0.7	17.4 ± 1.4
Thyroxine	0.3 ± 0.1	3.2 ± 0.5
Estrone	0.4 ± 0.1	83.9 ± 3.8
Dopamine	3.0 ± 1.2	0.3 ± 0.2
L-Dopa	ND	1.5 ± 0.3
D-Dopa	ND	2.6 ± 0.7
Dehydroepiandrosterone	0.2 ± 0.1	0.9 ± 0.1
p-Nitrophenol	10.1 ± 1.3	60.5 ± 4.4
2-Naphthylamine	16.9 ± 1.0	18.0 ± 0.4
2-Naphthol	122 ± 4	155 ± 4
Daidzein	13.1 ± 0.1	82.9 ± 3.5
Kaempferol	28.1 ± 3.2	91.2 ± 6.4
Caffeic acid	21.5 ± 1.4	12.1 ± 0.7
Genistein	6.8 ± 0.7	101 ± 3
Myricetin	19.3 ± 0.3	26.8 ± 3.6
Quercetin	80.5 ± 3.7	63.0 ± 2.8
Gallic acid	2.7 ± 1.1	4.0 ± 0.8
Chlorogenic acid	65.2 ± 4.2	4.7 ± 0.2
Catechin	58.8 ± 3.3	45.2 ± 4.2
Epicatechin	7.9 ± 0.4	17.1 ± 1.5
Epigallocatechin gallate	5.8 ± 1.6	6.5 ± 0.5
n-Propyl gallate	236 ± 11	66.9 ± 2.2
3-Chloro-4-biphenylol	153 ± 2	29.1 ± 0.6
3,3′,5,5′-Tetrachloro-4,4′-biphenyldiol	79.2 ± 1.9	11.1 ± 0.2

Table 3. Kinetic constants of zebrafish SULT1 STs 1 and 2 with hydroxychlorobiphenyls and 3,3',5-triiodo-L-thyronine as substrates. Data are given as mean ± SD from three experiments.

Substrate	SULT1 ST1			SULT1 ST2		
	K_m (μM)	V_{max} (nmol·min^{-1}·mg^{-1})	V_{max}/K_m	K_m (μM)	V_{max} (nmol·min^{-1}·mg^{-1})	V_{max}/K_m
3-Chloro-4-biphenylol	76.0 ± 7.7	435 ± 42	5.7	1.3 ± 0.1	66.7 ± 2.9	49.8
3,3',5,5'-Tetrachloro-4,4'-biphenyldiol	8.1 ± 1.0	145 ± 13	17.8	1.1 ± 0.1	18.1 ± 0.5	16.8
3,3',5-Triiodo-L-thyronine	64.4 ± 4.7	5.4 ± 0.1	0.08	9.4 ± 0.2	8.3 ± 0.2	0.9

higher V_{max}. That both of these enzymes displayed sulfating activities toward the two hydroxychlorobiphenyls may imply the utilization of sulfation as a means of inactivation/disposal of hydroxychlorobiphenyls in zebrafish.

Zebrafish are normally maintained in aquaria heated to 28 °C [24]. In their natural habitat, however, they are subjected to fluctuation in body temperature. An intriguing issue therefore is related to the stability of STs at different temperatures. A thermostability experiment was carried out in which the two zebrafish enzymes were first incubated for 15 min at different temperatures, followed by enzymatic assay under standard conditions with 2-naphthol as the substrate. As shown in Fig. 5, activity data obtained indicated that both zebrafish STs were stable over a relatively wide range of temperature (20–43 °C) under the experimental conditions used. At 48 °C, however, incubation for 15 min significantly lowered the activity of SULT1 ST1, while rendering SULT1 ST2 virtually inactive. Another issue is the effects of divalent metal cations on the activity of the zebrafish ST. Our previous studies had shown that divalent metal cations can exert dramatic inhibitory/stimulatory effects on various human cytosolic STs [25,26]. As an aquatic animal, zebrafish in the natural environment may be more vulnerable to the adverse effect of polluting heavy metal ions. Enzymatic assays using dopamine as the substrate were carried out in the absence or presence of various divalent metal cations at a concentration of 5 mM. As a control for the counter ion, Cl^-, parallel assays in the presence 10 mM NaCl were also performed. Results obtained are shown in Fig. 6. The degrees of inhibition or stimulation were calculated by comparing the activities determined in the presence of metal cations with the activities determined in the absence of metal cations. It was noted that NaCl control exerted only a marginal inhibitory effect on the activity of the zebrafish ST. Among 10 different divalent metal cations tested at 5 mM, Co^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} exhibited considerable inhibitory effects, while Hg^{2+} and Cu^{2+} rendered both enzymes virtually inactive. More detailed studies will be required in order to fully elucidate the dose-dependence of the regulation of the activity of the zebrafish ST by these divalent metal cations and their modes of action.

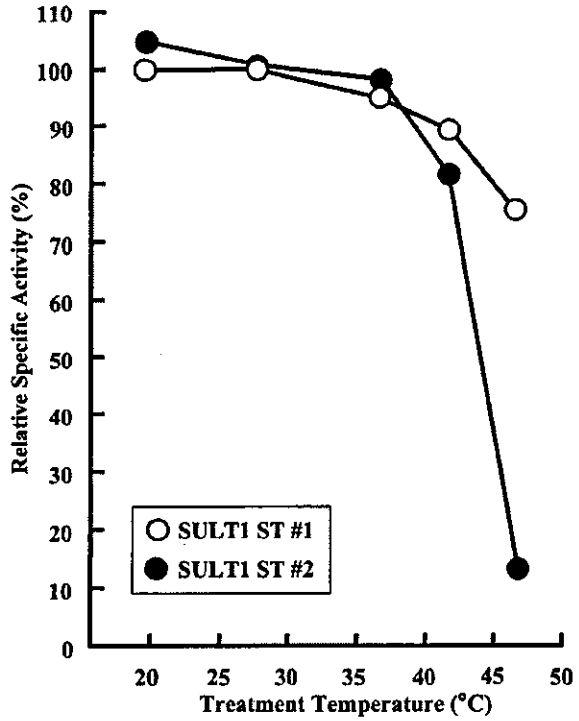

Fig. 5. Stability of zebrafish SULT1 STs 1 and 2 different temperatures. The relative activity of purified zebrafish ST incubated for 15 min at different temperatures is shown, followed by enzymatic assay using 2-naphthol as the substrate under standard conditions as described in Experimental procedures. The data represent calculated mean values derived from three experiments.

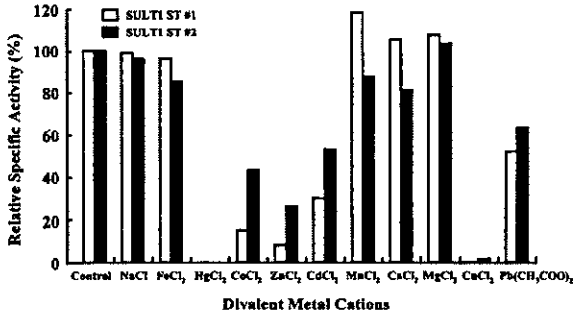

Fig. 6. Effects of divalent metal cations on the sulfating activity of the zebrafish SULT1 STs 1 and 2. Purified zebrafish ST was assayed for its dopamine-sulfating activity in the presence of different divalent metal cations or NaCl (as a control for the counter ion, Cl^-) under standard conditions as described in Experimental procedures. The concentration of the divalent metal cations tested was 5 mM, and the concentration of NaCl tested was 10 mM.

Expression of sebrafish SULT1 ST1 and SULT1 ST2 in cultured zebrafish liver cells and whole zebrafish

To examine the presence of mRNA encoding zebrafish SULT1 ST1 or SULT1 ST2, RT-PCR was used. As shown in Fig. 7A, a discrete PCR product (≈ 900 bp in size) corresponding to the SULT1 ST1 cDNA was found for both samples using the first-strand cDNA reverse-transcribed from the total RNA from either zebrafish liver cells (lane 1) or whole zebrafish (lane 2) as templates. A ≈ 900 bp PCR product corresponding to the SULT1 ST2 cDNA was also found for zebrafish liver cell sample (lane 3) and the whole zebrafish sample (lane 4). The authenticity of the PCR products corresponding to SULT1 ST1 and 2 cDNAs was confirmed by nested PCR using the primary PCR products as templates in conjunction with their respective 5′-primers and primers corresponding to sequences in the internal regions of SULT1 ST1 and 2 cDNAs (data not shown). These results indicated that, in zebrafish liver cells, both SULT1 ST1 and SULT1 ST2 mRNAs were expressed, with the latter being present at a considerably lower level than the former. Western blotting was then used to examine whether the zebrafish SULT1 ST1 protein is produced in cultured zebrafish liver cells. As shown in Fig. 7B, using rabbit antiserum against the zebrafish SULT1 ST1 as the probe, a distinct 35 kDa protein was detected, indicating clearly the production of the SULT1 ST1 protein in both cultured zebrafish cells and the whole zebrafish. Work is now in progress to examine in more detail the tissue-specific distribution of this enzyme.

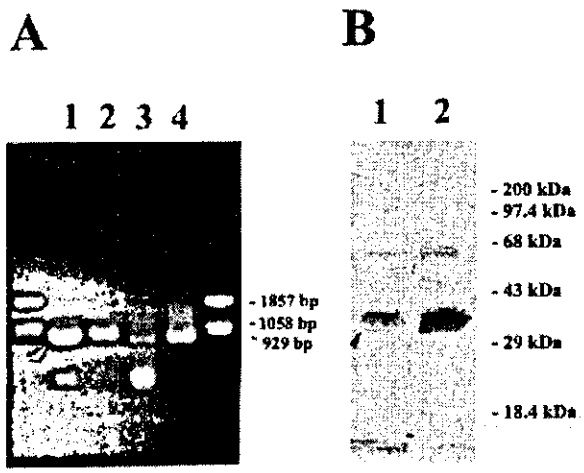

Fig. 7. (A) Detection of zebrafish SULT1 ST1 and ST2 mRNAs and (B) Western blot analysis of zebrafish SULT1 ST1 protein. (A) Detection of zebrafish SULT1 ST1 and ST2 mRNAs in cultured zebrafish cells (lanes 1 and 3) and whole zebrafish (lanes 2 and 4) by RT-PCR. The primers used for amplification of zebrafish SULT1 ST1 and 2 were the same as those listed in Table 1. DNA size markers coelectrophoresed during agarose electrophoresis are the *Mval*-restricted fragments of pBR322. The white arrowhead indicates the ≈ 900 bp PCR product band corresponding to SULT1 ST1 or ST2 cDNA. (B) Western blot analysis for the expression of zebrafish SULT1 ST1 protein in zebrafish liver cells (lane 1) and whole zebrafish (lane 2). Protein molecular mass markers: β-lactoglobulin (M_r = 18 400), carbonic anhydrase (M_r = 29 000), ovalbumin (M_r = 43 000), BSA (M_r = 68 000), phosphorylase *b* (M_r = 97 400), myosin (H-chain; M_r = 200 000). The black arrowhead indicates the 35 kDa protein band recognized by the antiserum against zebrafish SULT1 ST1.

Generation and release of [^{35}S]-sulfated hydroxychlorobiphenyls by zebrafish liver cells metabolically labeled with [^{35}S]sulfate

As mentioned previously, both SULT1 ST1 and SULT1 ST2 displayed strong enzymatic activities toward hydroxychlorobiphenyls (see Table 2). To examine whether sulfation of hydroxychlorobiphenyls occurs in a metabolic setting, confluent zebrafish liver cells, grown in individual wells of a 24-well culture plate, were incubated in sulfate medium containing [^{35}S]sulfate and 100 μM 3-chloro-4-biphenylol or 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl. At the end of a 12-h incubation, the media were collected for the analysis of [^{35}S]-sulfated products. As shown in Fig. 8, TLC revealed the presence of [^{35}S]-sulfated 3-chloro-4-biphenylol or 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl in the medium samples. These results demonstrated clearly the occurrence of the sulfation of 3-chloro-4-biphenylol and 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl in zebra-

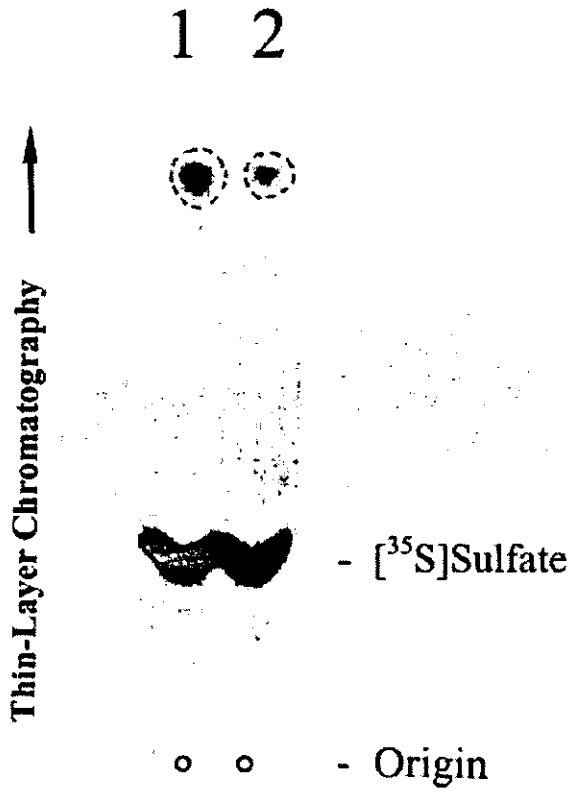

Fig. 8. Analysis of [^{35}S]-sulfated hydroxychlorobiphenyls generated and released by zebrafish liver cells labeled with [^{35}S]sulfate in the presence of hydroxychlorobiphenyls. The compounds tested were 3-chloro-4-biphenylol (lane 1) and 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl (lane 2). Dashed line circles indicate the corresponding [^{35}S]-sulfated hydroxychlorobiphenyls.

fish liver cells and the release of [^{35}S]-sulfated 3-chloro-4-biphenylol or 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl into the culture media.

In conclusion, the present study represents our new endeavour aimed at identifying the cytosolic ST enzymes present in zebrafish. As mentioned earlier, the identification of the various cytosolic STs followed by their biochemical characterization is a prerequisite for using zebrafish as a model for a systematic investigation of some of the fundamental and still unresolved questions regarding the role, ontogeny, and regulation of the cytosolic STs. More work is definitely warranted in order to achieve this goal.

Acknowledgements

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「硫酸転移酵素を用いた変異原試験法における食品の機能性評価に関する研究」

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はじめに

生体内における硫酸化は，チトクロームP450酵素群の行うフェーズⅠ解毒代謝としての酸化還元反応に対し，グルクロン酸抱合などと同様フェーズⅡ反応に分類される。硫酸化は生体外異物や薬物の解毒代謝への関与の他に，内因性の化合物であるステロイドホルモンや甲状腺ホルモン，神経伝達物質であるカテコールアミンの代謝にまで重要な関わりを持つことが知られている[1]。

生体内での硫酸化にはまず硫酸の活性化，即ち硫酸基供与体である活性硫酸PAPS（3'-phosphoadenosine 5'-phosphosulfate）の生合成が必要である。PAPSは1950年代にLipmannらにより発見され，ATPと無機硫酸から合成されることが知られている[2]（図1）。このPAPS合成には2種の酵素活性即ちATP sulfurylaseとadenosine 5'-phosphosulfate kinase（APS kinase）によって触媒される。これら二つのPAPS合成に関与する酵素は，大腸菌やかびといった微生物や植物においては二つの異なるタンパク質として存在している。しかしヒトやマウスなどの哺乳動物やショウジョウバエにおいては，二つの酵素が進化の過程で融合したPAPS synthetase（bifunctional ATP sulfurylase/adenosine 5'-phosphosulfate kinase）として存在し，現在ヒトとマウスではPAPS synthetase 1とPAPS synthetase 2の2種が確認されており，より効率よくPAPSが合成できるように進化したと考えられている。

$$ATP + SO_4^{2-} \underset{\text{ATP sulfulylase}}{\rightleftharpoons} APS + PPi$$

$$APS + ATP \xrightarrow[\text{APS kinase}]{} PAPS + ADP$$

図1　活性硫酸PAPS（3'-phosphoadenosine 5'-phosphosulfate）の合成

生体内における低分子化合物の硫酸化は硫酸転移酵素と呼ばれる酵素群によって触媒され，その種類も1997年当時の硫酸転移酵素に関する総説においては，ヒトにおいてフェノール硫酸転移酵素2種，ヒドロキシステロイド硫酸転移酵素1種，エストロゲン硫酸転移酵素1種の合計4種の酵素の存在が知られているにすぎなかった[3]。しかし現在では様々な動物種において硫酸転移酵素のクローニングが盛んに行われ，新規の硫酸転移酵素が多数発見され，ヒトで10種，マウスにおいては14種発見されている。これら硫酸転移酵素の分類に関しては硫酸転移酵素ワークショップにおいて提唱された分類法により，アミノ酸配列をもとに分類することが推奨されている。硫酸転移酵素遺伝子は*SULT*（遺伝子の場合イタリック）という略語を用いて，その後にアミノ酸配列が30%以上一致するファミリーを表す数字を付ける。例えば，*SULT1*はフェノール硫酸転移酵素ファミリー，*SULT2*はヒドロキシステロイド硫酸転移酵素ファミリー，*SULT3*はアミン硫酸

転移酵素ファミリーというように分類される。さらにこれらのファミリーごとにアミノ酸配列が60%以上一致するグループをサブファミリーとし，アルファベットをAから順に付けていく。例えば，P型フェノール硫酸転移酵素については*SULT1A1*と*SULT1A2*の存在が確認されている。

生体内における硫酸化の役割は，前述したように薬物など生体外異物の水溶性を高め，尿中に溶けやすくし体外へ排出することと，ホルモン類や神経伝達物質といった内因性の生理活性物質の濃度調節機構であり，これらは体内における恒常性を維持するための重要で不可欠な働きである。しかしながら，時には硫酸化がその個体において悪影響を及ぼす場合がある。即ち，硫酸化が変異原物質の代謝活性化に関与している事実である。硫酸化によって変異原性を示す化合物はいくつか報告されており，N-ヒドロキシ-2-アセチルアミノフルオレンやヒドロキシメチルピレンなどがある[4) 5)]。今回の実験において用いた9-ヒドロキシメチルアントラセン（9HMA）もその一つであり，汚泥中や魚類などから発見されている。

1．硫酸転移酵素を用いた変異原試験法の確立

成人病の中で日本の死因の1位を占めるガンは，その原因のほとんどが環境因子によるものだとされている。ディーゼルの排出ガスやタバコの煙，食品中のアミノ酸の加熱分解産物であるTrp-P-1やTrp-P-2，Glu-P-1など生活環境の中には様々な化学発癌物質が存在している。これら発癌の疑いのある化合物をあらかじめスクリーニングしておくことは非常に重要であり，我が国でも新規の化合物についてはその変異原性，発ガン性の可能性を試験することが義務付けられている。その試験としてAmes試験が世界中で広く普及している。この試験は改良された*Salmonella typhimurium* TAシリーズを用いて，ヒスチジン要求性から非要求性へと変わる復帰突然変異を，効率よく簡便にプレート上で検出するものである。また変異原物質の多くは動物の体内で代謝活性

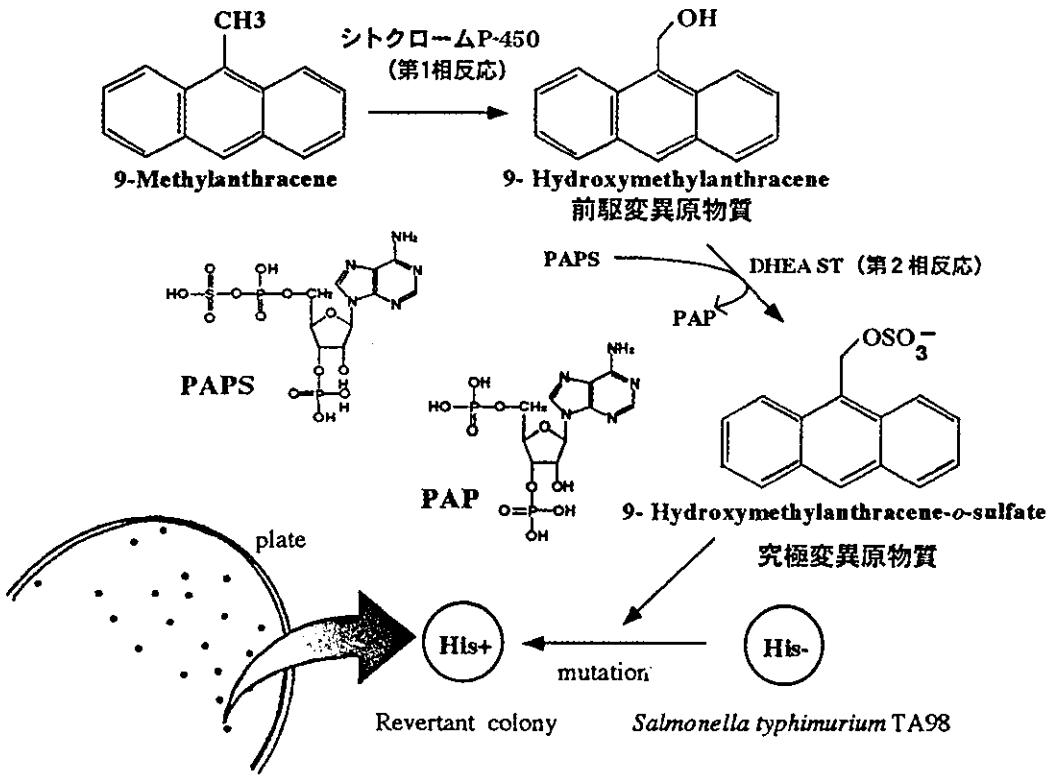

図2　9HMAの代謝活性化

化されて初めて変異原性を示す化合物であることから，その目的のためにラットの肝臓から調製したS9という酵素画分を用いることが特徴である。この画分には，動物体内のフェーズⅠ解毒代謝機構である酸化還元反応を行うチトクロームP450酵素群が多く含まれている。では，この試験法を用いて体内で起こるフェーズⅡ解毒代謝によって代謝活性化され変異原性を増す化合物の検出は可能だろうか。そこで我々はフェーズⅡ反応を行う硫酸転移酵素を用いて変異原試験法を確立した（図2）。本実験ではアミノ酸配列をもとにする分類法で*SULT2A1*に属するヒドロキシステロイド系の酵素（デヒドロエピアンドロステロン硫酸転移酵素；DHEA ST）を用いた。9-メチルアントラセンはフェーズⅠ反応により酸化され9HMAになる。Ames試験においては，反応はここで終了するであろう。しかし前述したように，この化合物こそ硫酸化によって代謝活性化され究極変異原物質に変わる前駆変異原物質である。確立した試験により，9HMAは硫酸化による代謝活性化を受け，硫酸転移酵素濃度及びPAPS濃度依存的に復帰突然変異コロニー数が増加した（図4A）。尚，9HMAをAmes法によるS9 mixを用いて実験を行った結果，大きな変異原性は確認できなかった。厳密にはS9中にもいくつかの硫酸転移酵素は存在するためやや検出できるが，酵素画分に含まれるPAPSの安定性が悪いため検出が弱いと考えられる。

この試験法の確立により，今まで変異原性を示さないと考えられていた化合物で硫酸化によって代謝活性化される化合物を特異的に検出できるようになるであろう。

2．食品の機能性評価

生活の豊かさや欧米食の普及による食生活の変化に伴い増加の一途をたどる成人病をはじめ，様々な生活習慣病に対し，近年人々の意識の中に強い健康志向が高まっている。中でも食品の機能性に注目が集まっており，ここ数年，フレンチ・パラドクスなどと言われ注目を集めた赤ワインにおいては，その色を付けるブドウの皮に含まれているタンニン，アントシアニンなどのポリフェノール類が体にいいようである。また，昔から日本人が飲み続けているお茶の中にもカテキンなどの多くのポリフェノールが含まれ，ガン予防をはじめとする多くの効能が知られている。ポリフェノールとはA環の5,7位やB環の3',4'位にヒドロキシル基を持つことから言われ(図3)，抗酸化性をはじめ，サイトカイン産生の促進と抑制による免疫調節，アゴニストやアンタゴニストとしてのホルモン作用調節，癌細胞の増殖抑制やアポトーシス誘導と正常細胞への分化誘導作用，そして制菌，殺菌作用などがある。

私達は緑茶由来のカテキン類8種において，その機能性としての抗変異原性について硫酸転移酵素を用いた変異原試験法で検討した。その結果，没食子酸を持つガロカテキンガレート（GCG），エピカテキン-3-*O*-ガレート（ECG），エピガロカテキン-3-*O*-ガレート（EGCG）（図4B）が最も強い抗変異原作用を示し，その抗変異原性は硫酸転移酵素による変異原物質の代謝活性化への競合阻害による可能性が示唆された。更に，EGCGのメチル体を用いてその構造から検討した結果，B環および没食子酸の4位のヒドロキシル基が重要である可能性が示唆された。

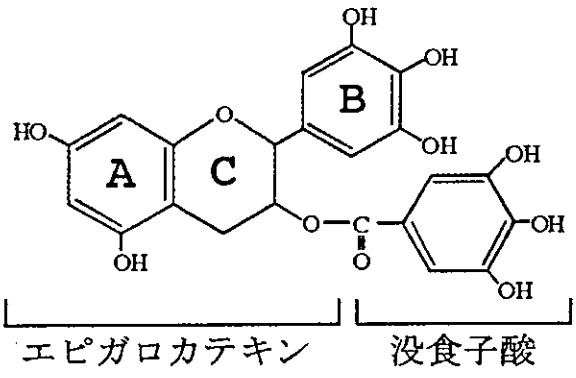

図3　エピガロカテキン-3-*O*-ガレート(EGCG)の構造

EGCGについては多くの研究報告があり，その

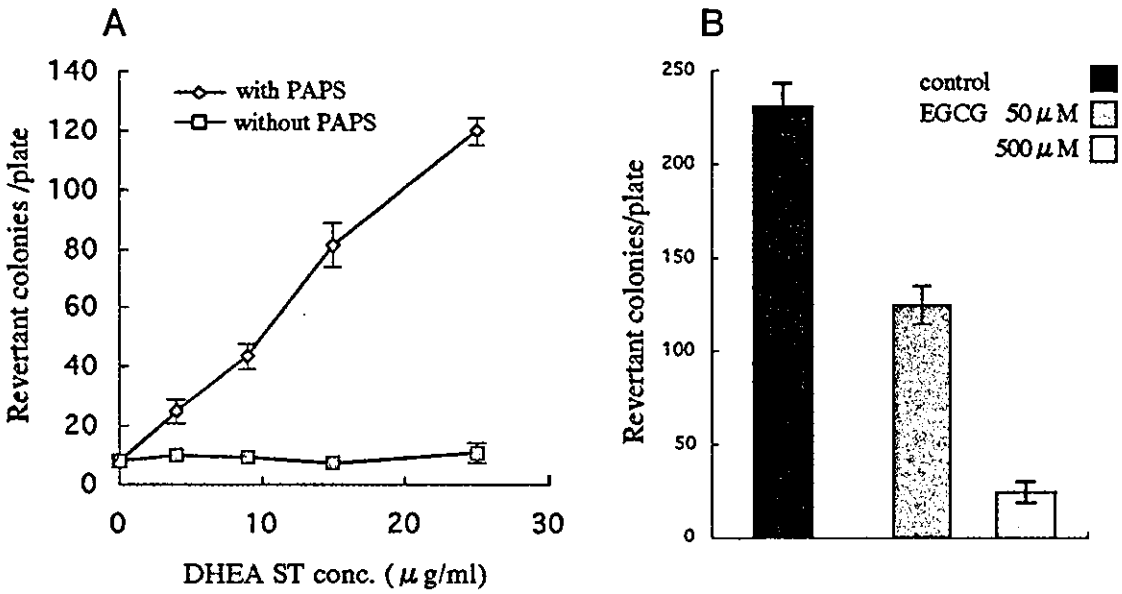

図4　酵素濃度依存性とEGCGの抗変異原性

生理活性が他のフラボノイドより高いことが分かっている。EGCGは緑茶1～2杯の中におよそ100mgも含まれているとされており，ヒトの健康維持に高く貢献していると考えられる。また我々は大豆成分であるイソフラボン類においても抗変異原性を確認しており，今後も食品の機能性に高く注目していきたい。

3．これからの展望

1953年にワトソン・クリックがDNAの二重らせん構造を提唱した事から，今年は遺伝子の夜明けから50年といわれており，その節目にヒトゲノムの完全解読完了宣言がなされた。そのヒトゲノム計画の成果で最も期待されているのが，医学への応用である。現在の医療においてはある病気が発症してから治療が始まるが，将来的にはある病気にかかりやすい体質と判断された時点から，発病しないような処置を施す予防医療の実現が大きな目標とされる。近年はポストゲノム時代と呼ばれ，個人の体質を決定している遺伝子の違いが着目されている。遺伝子の中でたった一つの塩基配列の違いが病気の原因となったり，薬の効果を左右したりする。この様な塩基配列の違いを一塩基多型（SNP: single nucleotide polymorphism）と呼び，このSNP解析こそが，ポストゲノム研究において最も注目されている分野である。将来的にこういった個人個人に見合った治療法，テーラーメイド医療が期待されている。日本の死因で1位を占めるガンにおいても，この治療法が適用できれば遺伝的な背景から発ガンリスクを予測し，日常生活の改善による予防も可能となってくると期待されている。

薬物代謝酵素の一つである硫酸転移酵素もまた格好のテーラーメイド医療の対象として，投薬量の決定すなわち薬のさじ加減に大きく関わると考えられて精力的に研究されている。今回の試験で用いた硫酸転移酵素についても何種類かのSNPが確認されており，前駆変異原物質の代謝活性化能力の違いと，食品機能性成分の抗変異原作用への影響について検討していくことが最も興味の惹かれるところである。

おわりに

現在，硫酸転移酵素を*Salmonella typhimurium* TAシリーズやV79細胞などの動物細胞に組み込み，変異原試験や抗酸化試験などを行った報告がいくつかみられる[5) 6) 7)]。今回，我々は Hansruedi Glatt等が1995年に報告した論文[8)]を参考に，細胞外で硫酸化され究極変異原物質となる9-ヒドロキシメチルアントラセンをモデル変異原物質とした変異原試験法を確立し，食品の機能性を評価してきた。今後，コンビナトリアルケミストリーによる技術の進歩に伴いますます増加すると考えられる合成化学薬品に対し，体内で起こりうる様々な代謝活性化反応を考慮して試験していく必要が考えられる。そしてまだまだ未開拓の食品の機能性に関する分野で新たな可能性に期待し研究を続けていきたいと考える。

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Sulfonation of environmental estrogens by zebrafish cytosolic sulfotransferases☆

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Abstract

Environmental estrogen-like chemicals are increasingly recognized as a potential hazardous factor for wildlife as well as humans. We have recently embarked on developing a zebrafish model for investigating the role of sulfonation in the metabolism and adverse functioning of environmental estrogens. Here, we report on a systematic investigation of the sulfonation of representative environmental estrogens (bisphenol A, 4-*n*-octylphenol, 4-*n*-nolylphenol, diethylstilbestrol, and 17α-ethynylestradiol) by zebrafish cytosolic sulfotransferases (STs). Of the seven enzymes tested, four zebrafish STs (designated ZF ST #2, ZF ST #3, ZF ST #4, and ZF DHEA ST) exhibited differential sulfonating activities toward the five environmental estrogens tested, with ZF ST #3 being more highly active than the other three. It was further demonstrated that bisphenol A, 4-*n*-octylphenol, and 4-*n*-nonylphenol exerted concentration-dependent inhibition of the sulfonation of 17β-estradiol, implying a potential role of these environmental estrogens in interfering with the sulfonation, and possibly homeostasis, of endogenous estrogens. Kinetic studies revealed that the mechanism underlying the inhibition by bisphenol A or 4-*n*-nonylphenol to be of the competitive type.

Sulfotransferases (STs) are enzymes, present in both plants and animals, that catalyze the sulfonation of a variety of compounds containing hydroxyl or amino groups, using 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the sulfonyl group donor [1–3]. While the membrane-bound STs use proteins, glycolipids, and other macromolecules as substrates, the cytosolic STs sulfate low-molecular weight endogenous and xenobiotic compounds. This serves to both regulate the levels and activities of endogenous molecules such as thyroid/steroid hormones and catecholamine hormones/neurotransmitters, as well as detoxify dietary, therapeutic, and environmental xenobiotics [4,5].

In recent years, environmental estrogen-like chemicals have been increasingly recognized as a potential hazardous factor for wildlife as well as humans [6]. In general, they are able to bind to estrogen receptors and thereby mimic estrogenic actions [7] or interfere with the action of enzymes which help regulate the level of endogenous estrogens and other hormones [8]. Some examples of this diverse group of compounds are diethylstilbestrol, bisphenol A, nonylphenol, polychlorinated biphenyls, and dichlorodiphenyltrichloroethane [9]. These environmental estrogens are becoming ubiquitous in the environmental and making their way into the food chain. Among other adverse effects, the environmental estrogens have been implicated in the abnormal sexual development of reptiles and bird [10,11], the decline in sperm quality of men [12,13], and an increased incidence of human breast cancer [14,15]. We have recently demonstrated the sulfonation of some environmental estrogens by human cytosolic STs [16,17]. The role of sulfonation in the metabolism and

☆ *Abbreviations:* ST, sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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modulation of environmental estrogens in the context of physiology, however, remains unknown. To obtain such information, a suitable animal model is required.

Zebrafish has in recent years emerged as a popular animal model for a wide range of studies [18,19]. Its advantages, compared with mouse, rat, or other vertebrate animal models, include the small size, availability of relatively large number of eggs, rapid development externally of virtually transparent embryo, and short generation time. These unique characteristics of the zebrafish make it an excellent model for a systematic investigation on the physiological involvement of cytosolic STs, including the sulfonation of environmental estrogens. A prerequisite for using zebrafish in these studies, however, is the identification of the various cytosolic STs and their functional characterization. We have recently embarked on the molecular cloning of zebrafish cytosolic STs, and have expressed and purified a number of these enzymes [20–22].

In this communication, we report on the characterization of the sulfonating activities of zebrafish cytosolic STs toward representative environmental estrogens. Kinetic parameters of the sulfonation of these environmental estrogens were determined. Moreover, the inhibitory effects of these compounds on the sulfonation of 17β-estradiol, an endogenous estrogen, and the underlying mechanism were studied.

Materials and methods

Materials. Bisphenol A, diethylstilbestrol, 4-*n*-octylphenol, 4-*n*-nonylphenol, 17α-ethynylestradiol, 17β-estradiol, dehydroepiandrosterone (DHEA), estrone, aprotinin, thrombin, adenosine 5′-triphosphate (ATP), PAPS, sodium dodecyl sulfate (SDS), *N*-2-hydroxylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), Trizma base, isopropyl β-D-thiogalactopyranoside (IPTG), inorganic pyrophosphatase, and dithiothreitol (DTT) were obtained from Sigma Chemical. Human ATP sulfurylase/APS kinase was prepared as described previously [23]. BL21 (DE3) *Escherichia coli* host strain was from Novagen. Carrier free sodium [^{35}S]sulfate was from ICN Biomedicals. Cellulose thin-layer chromatography (TLC) plates were from EM Science. All other reagents were of the highest grade commercially available.

Expression and purification of recombinant zebrafish cytosolic STs. We have previously cloned seven zebrafish cytosolic ST cDNAs, packaged them individually in pGEX-2TK or pET23c prokaryotic expression vector, and transformed the plasmid constructs into BL21 (DE3) cells [20–22]. Transformed BL21 (DE3) cells were grown in 1 L of LB broth containing 50 μg/mL ampicillin. After the cell density reached 0.7 OD_{600nm}, 0.1 mM (for pGEX-2TK constructs) or 0.4 mM (for pET23c constructs) IPTG was added to induce the production of recombinant zebrafish cytosolic ST. After a 5-h induction at 37 °C, the cells were collected by centrifugation and homogenized in 20 mL of a lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) using an Aminco French Press. Recombinant zebrafish cytosolic STs present in the cell homogenates were purified using previously established procedures [20–22], analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), determined for protein concentration, and used in the enzymatic assays.

Enzymatic assay. The ST assays were performed using PAP[^{35}S] as the sulfonyl group donor. The standard assay reaction mixture contained, in a final volume of 25 μL, 50 mM Hepes, pH 7.0, 14 μM PAP[^{35}S], and 50 μM substrate. The reaction was started by the addition of enzyme (0.025 μg), allowed to proceed for 3 min at 28 °C, and stopped by heating at 100 °C for 2 min. Upon brief centrifugation to pellet down the precipitates, 1 μL aliquot of the reaction mixture was spotted on a cellulose plate for the TLC analysis of [^{35}S]sulfated product based on our previously established procedure [24]. For the kinetic studies on the sulfonation of endogenous estrogens (estrone and 17β-estradiol) and environmental estrogens, varying concentrations (ranging from 0.5 to 50 μM) of these substrate compounds and 50 mM Hepes at pH 7.0 were used. To determine the inhibitory effects of bisphenol A, 4-*n*-octylphenol, and 4-*n*-nonylphenol, enzymatic assays in the presence of varying concentrations (ranging from 0 to 1 mM) of these environmental estrogens were performed with 50 μM 17β-estradiol as substrate. To investigate the mechanism underlying the inhibition of the sulfonation of 17β-estradiol by bisphenol A or 4-*n*-nonylphenol, enzymatic assays using varying concentrations (5–20 μM) of 17β-estradiol in the presence of fixed concentrations of bisphenol A (0, 400, and 800 μM) or 4-*n*-nonylphenol (0, 20, and 50 μM) were performed.

Miscellaneous methods. PAP[^{35}S] (carrier-free) was synthesized from ATP and carrier-free [^{35}S]sulfate using the human bifunctional ATP sulfurylase/APS kinase as described previously [23]. The PAP[^{35}S] synthesized was then adjusted to the desired concentration and specific activity by the addition of cold PAPS. SDS–PAGE was performed on a 12% polyacrylamide gel using the method of Laemmli [25]. Protein determination was based on the method of Bradford with bovine serum albumin as the standard [26].

Results and discussion

In view of the mounting evidence of the hazardous effects of environmental estrogens on both wildlife and humans [6,10,11], we became interested in investigating whether sulfonation, a Phase II detoxification pathway, might be involved in the metabolism and modulation of these compounds. In our earlier studies [16,17], we demonstrated indeed the occurrence of the sulfonation of representative environmental estrogens by human cytosolic STs. We recently embarked on developing the zebrafish as an animal model for investigating the sulfonation of environmental estrogens in the context of physiology. We started by cloning the various cytosolic STs present in zebrafish [20–22]. The seven distinct zebrafish cytosolic STs cloned to date were bacterially expressed and examined with respect to their environmental estrogen-sulfonating activities, as well as the inhibitory effects of environmental estrogens on the sulfonation of an endogenous estrogen, 17β-estradiol.

Preparation and characterization of zebrafish cytosolic STs with environmental estrogens as substrates

The seven zebrafish cytosolic STs previously cloned [20–22] were bacterially expressed and purified. Fig. 1 shows the SDS–gel electrophoretic pattern of the purified zebrafish cytosolic STs. These purified STs were first assayed for sulfonating activity towards some typical environmental estrogens (including bisphenol A, 4-*n*-oc-

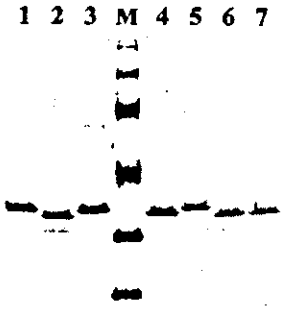

Fig. 1. SDS–gel electrophoretic patterns of purified zebrafish cytosolic STs. Samples analyzed in lanes 1–7 were: ZF ST #1, ZF ST #2, ZF ST #3, ZF ST #4, ZF ST #5, ZF DHEA ST, and ZF NST. Protein molecular weight markers co-electrophoresed in lane M are β-lactoglobulin (M_r = 18,400), carbonic anhydrase (M_r = 29,000), ovalbumin (M_r = 43,000), bovine serum albumin (M_r = 68,000), phosphorylase b (M_r = 97,400), and myosin (H-chain) (M_r = 200,000).

tylphenol, 4-*n*-nonylphenol, diethylstilbestrol, and 17α-ethynylestradiol) and an endogenous estrogen, 17β-estradiol. In these experiments, a saturating concentration of PAPS, i.e., 14 and 50 μM of environmental estrogens or 17β-estradiol were used in the assays. The results compiled in Table 1 showed that three of the seven zebrafish STs (ZF ST #1, ZF ST #5, and ZF NST) were inactive. The other four zebrafish STs (ZF ST #2, ZF ST #3, ZF ST #4, and ZF DHEA ST) exhibited differential sulfonating activities toward the five environmental estrogens tested, with ZF ST #3 being more highly active than the other three. We therefore decided to examine in greater detail the kinetic parameters of ZF ST #3 in catalyzing the sulfonation of these environmental estrogens and 17β-estradiol. In these experiments, varying concentrations of environmental estrogens or 17β-estradiol were used in the assays. The kinetic constants determined are compiled in Table 2. Of the five environmental estrogens tested, 4-*n*-octylphenol and diethylstilbestrol appeared to be better substrates for the enzyme, as reflected by the calculated V_{max}/K_m values.

An important issue is whether and how the presence of environmental estrogens may interfere with the sulfonation of endogenous estrogens. To resolve this issue,

Table 2
Kinetic constants of ZF ST #3 with environmental estrogens and endogenous estrogens as substrates[a]

Substrate	V_{max} (nmol/min/mg)	K_m (μM)	V_{max}/K_m
Bisphenol A	15.4	31.1	0.5
4-*n*-Octylphenol	356	18.5	19.2
4-*n*-Nonylphenol	158	27.3	5.8
Diethylstilbestrol	208	9.0	23.1
17α-Ethynylestradiol	136	16.1	8.4
Estrone	366.4	12.5	29.3
17β-Estradiol	175.4	15.1	11.6

[a] Data shown represent mean values derived from three determinations.

we first tested the inhibitory effects of environmental estrogens on the sulfonation of 17β-estradiol. Enzymatic assays were carried out using 5 μM 17β-estradiol as substrate in the presence of different concentrations (ranging from 0 to 1 mM) of bisphenol A, 4-*n*-octylphenol, or 4-*n*-nonylphenol. As shown in Fig. 2, all three environmental estrogens tested exerted concentration-dependent inhibition of the sulfonation of 17β-estradiol. The IC_{50} values determined based on the results shown in the figure were 90, 5, and 17.5 μM, respectively, for bisphenol A, 4-*n*-octylphenol, and 4-*n*-nonylphenol. These results indicated clearly the potential role of environmental estrogens in interfering with the sulfonation, and possibly homeostasis, of endogenous estrogens.

To further investigate the mechanism underlying the inhibition of 17β-estradiol sulfonation by environmental estrogens, enzymatic assays using varying concentrations (5–20 μM) of the substrate, 17β-estradiol, in the presence of fixed concentrations of bisphenol A (0, 400, and 800 μM) or 4-*n*-nonylphenol (0, 20, and 50 μM) were performed. Data obtained were used to generate the Lineweaver–Burk double-reciprocal plots. As shown in the double reciprocal plots generated (Fig. 3), the lines corresponding to the various concentrations of bisphenol A (Part A) or 4-*n*-nonylphenol (Part B) tested, while crossing the *X*-axis at different positions, appeared to converge within a narrow region on the *Y*-axis. These

Table 1
Specific activities of zebrafish cytosolic STs with environmental estrogens as substrates[a]

Specific activity (nmol/min/mg)							
Substrate	ZF ST #1	ZF ST #2 (SULT1 ST#1)	ZF ST #3 (SULT1 ST #2)	ZF ST #4	ZF ST #5	ZF DHEA ST (SULT2 ST)	ZF NST
Bisphenol A	ND[a]	ND	7.6 ± 0.4	1.7 ± 0.3	ND	ND	ND
4-*n*-Octylphenol	ND	51.7 ± 5.0	68.2 ± 5.3	74.7 ± 2.4	ND	ND	ND
4-*n*-Nonylphenol	ND	7.0 ± 0.5	34.9 ± 2.6	19.4 ± 0.6	ND	0.6 ± 0.1	ND
Diethylstilbestrol	ND	0.6 ± 0.3	51.4 ± 5.8	8.6 ± 1.0	ND	ND	ND
17α-Ethynylestradiol	ND	ND	61.7 ± 1.5	2.0 ± 0.3	ND	ND	ND
17β-Estradiol	ND	ND	79.0 ± 4.0	2.4 ± 0.6	ND	0.6 ± 0.1	ND

[a] ND, activity not detected. Data shown represent means ± SD derived from three determinations.

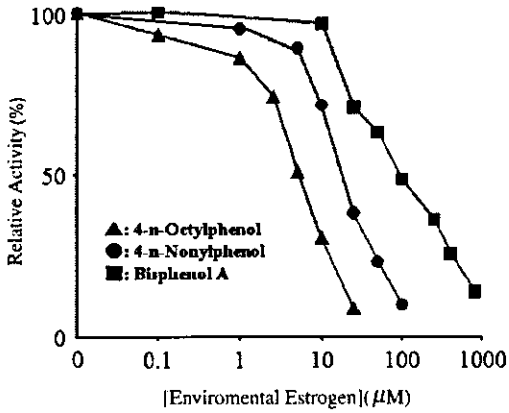

Fig. 2. Inhibition of 17β-estradiol sulfonation by 4-*n*-octylphenol (▲), 4-*n*-nonylphenol (●), and bisphenol A (■). Enzymatic assays in the presence of varying concentrations of these environmental estrogens were performed with 50 μM 17β-estradiol as substrate. Data were calculated based on the activity determined in the absence of environmental estrogen as 100%.

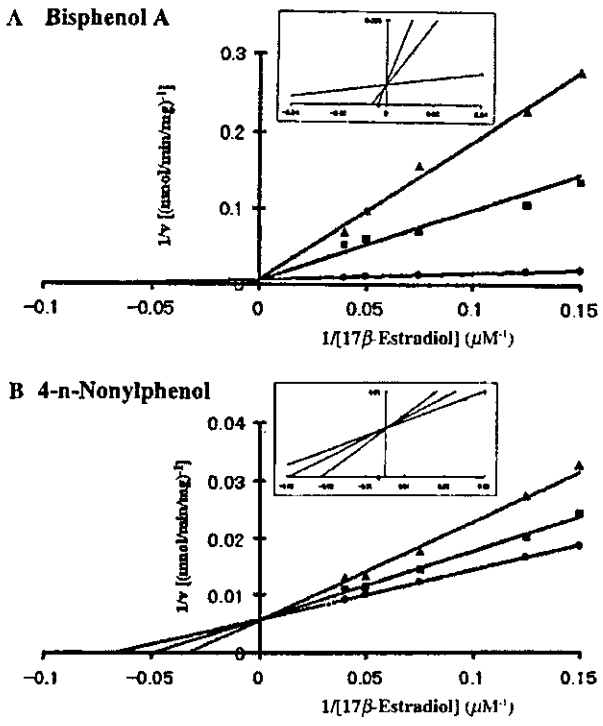

Fig. 3. (A) Lineweaver–Burk double-reciprocal plot of ZF ST #3 with 17β-estradiol as substrate in the presence of different concentrations of bisphenol A. Concentrations of bisphenol A tested were: 0 μM (●), 400 μM (■), and 800 μM (▲). (B) Lineweaver-Burk double-reciprocal plot of ZF ST #3 with 17β-estradiol as substrate in the presence of different concentrations of 4-*n*-nonylphenol. Concentrations of 4-*n*-nonylphenol tested were: 0 μM (●), 20 μM (■), and 50 μM (▲). Concentrations of 17β-estradiol are expressed in μM and velocities are expressed as nmol of product formed/min/mg enzyme. Each data point represents the mean value derived from three determinations.

Table 3
Kinetic constants of ZF ST #3 at different concentrations of bisphenol A or 4-*n*-nonylphenol with 17β-estradiol as substrate[a]

Inhibitor concentration (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max}/K_m
Bisphenol A			
0	175.4	15.1	11.6
400	175.4	162.1	1.1
800	158.7	286.5	0.6
4-n-Nonylphenol			
0	175.4	15.1	11.6
20	172.4	20.4	8.5
50	178.6	30.6	5.8

[a] Data shown represent mean values derived from three determinations.

results indicated that the V_{max} value of the zebrafish EST for 17β-estradiol did not change much in the presence of bisphenol A or 4-*n*-nonylphenol. Whereas the K_m increased dramatically with increasing concentrations of bisphenol A or 4-*n*-nonylphenol. K_m and V_{max}, as well as V_{max}/K_m, calculated from the Lineweaver–Burk double reciprocal plots are compiled in Table 3. These data imply likely a competitive-type of inhibition.

In conclusion, we report in this paper the sulfonation of some representative environmental estrogens by zebrafish cytosolic STs, and the inhibitory effects of these compounds on the sulfonation of 17β-estradiol, an endogenous estrogen. These studies provide a basis for further investigation into the role of sulfonation in the metabolism and adverse functioning of environmental estrogen using the zebrafish as a model.

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