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Immunological Characterization of Human Phenol Sulfotransferase

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SUMMARY

The immunological characterization of the different forms of phenol sulfotransferase (PST) in a variety of human and nonhuman tissues is described. Immunoblotting techniques revealed that polyclonal antibodies raised to human platelet M_{II}-PST reacted with polypeptides of 32 and 34 kDa from human platelet 100,000 × *g* supernatant solution. Immunoblot analysis of platelet 100,000 × *g* supernatant solution that was fractionated over a DEAE-cellulose column indicated a close correspondence of P-PST activity, as measured by phenol sulfation, and M-PST activity, as assessed by dopamine sulfation, with the 32 and 34 kDa polypeptides, respectively. Examination of various human tissues revealed the presence of immunologically detectable levels of P-PST in liver and adrenal gland whereas both M- and P-PST were detected in placenta at a 1/10,000 dilution of the antisera. Under these conditions, PST was undetectable in human frontal cortex, pituitary gland, kidney, lung, and jejunum. Further evaluation of human liver samples from four individuals indicated a strong correlation ($r = 0.94$) between the amount of 32-kDa immunoreactive protein and P-PST activity. Analysis of liver samples from several animal species (monkey, rat, mouse, guinea pig, and frog) revealed the presence of immunoreactive proteins of various molecular masses, suggesting that considerable homology may exist between human and nonhuman forms of PST.

Sulfoconjugation is a significant phase II drug-metabolizing pathway for the processing of several therapeutically relevant phenolic drugs (1) as well as the catecholamine neurotransmitters (2). The enzymes responsible for the sulfation of this structurally diverse group of catechol and phenolic compounds are classified as PSTs (EC 2.8.2.1). These enzymes have been extensively studied during the past decade and considerable information regarding their biochemical and kinetic properties has been obtained (3). Human PST activity has been categorized into two functionally distinct classes, termed M-PST and P-PST, that are primarily distinguished by their substrate specificities (4) as well as differences in their sensitivity to temperature (5) and inhibition by dichloro-p-nitrophenol (6). The M form of human PST is so termed because of its selective sulfation of dopamine and other structurally related monoamines, whereas the P form preferentially sulfoconjugates phenol. In addition, M-PST is more heat labile (5) and less sensitive to inhibition by dichloro-p-nitrophenol than P-PST (6).

The number of protein species responsible for M- and P-PST activities, as well as their distribution in human tissues, is not well understood. Recent data from our laboratory demonstrate the presence of two discernable forms of both M- and P-PST activity in human brain (7) and platelets (8) by anion exchange chromatography of 100,000 × *g* supernatant solutions of these tissues. The most electronegative form of the human platelet enzyme, termed M_{II}-PST, has recently been purified in our laboratory (8) and shown to exist as a homodimer with apparent subunit and native molecular masses of approximately 34 and 69 kDa, respectively.

To better understand the tissue-specific expression of human M_{II}-PST and possibly other forms of PST, we report here on the use of polyclonal antibodies directed against purified human platelet M_{II}-PST to immunologically identify different forms of PST in several human and nonhuman tissues.

Experimental Procedures

Materials. Goat anti-rabbit immunoglobulin G (H plus L)-alkaline phosphatase was purchased from Bio-Rad (Rockville Centre, NY). Freund's adjuvant was obtained from Difco Laboratories (Detroit, MI). Nitrocellulose paper was acquired from Schleicher & Schuell (Keene, NH). PAPS was obtained from Sigma Chemical Company (St. Louis, MO) and Whatman DE52 anion exchanger was purchased from Krackler Scientific (Albany, NY). [^{35}S]PAPS (specific activity, approximately 1.1 Ci/mmol) was acquired from New England Nuclear (Boston, MA). Outdated human platelets were purchased from the American Red

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ABBREVIATIONS: PST, phenol sulfotransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; TBS, Tris-buffered saline.

Cross (Rochester, NY) and fresh human liver specimens were obtained from the Organ Procurement Program, Strong Memorial Hospital, University of Rochester. Purified rat liver arylsulfotransferase IV was a generous gift of Dr. Michael Duffel, University of Iowa.

Tissue preparation. Human lung, jejunum, kidney, and pituitary gland were obtained at the time of autopsy, usually within 20 hr from the time of death. These tissues and human platelets were each homogenized for 10 to 20 sec with a Brinkman Polytron, whereas frontal cortex and liver samples were disrupted for a similar amount of time with a motorized glass-Teflon homogenizer. Homogenizations were performed in 5 ml of 10 mM triethenolamine, pH 7.4, containing 0.25 M sucrose and 5 mM β-mercaptoethanol per g of tissue. The crude homogenates were centrifuged at 12,000 × *g* for 30 min and the resulting supernatant solutions were centrifuged for an additional 60 min at 100,000 × *g*.

PST assay. PST activity was determined as described previously (7). In brief, reaction mixtures routinely consisted of 1 μM radiolabeled PAPS, 50 mM triethanolamine buffer, pH 7.4, and 10 μM concentrations of either dopamine or phenol for the assay of M-PST and P-PST activity, respectively. The Ecteola cellulose chromatographic method (9) was used to separate radiolabeled products from reactants.

Immunization procedure. Human platelet M_{II}-PST was purified to electrophoretic homogeneity using DEAE-cellulose, Sephacryl S-200 HR, and 3′-phosphoadenosine-5′-phosphate-agarose chromatography (8). For the production of antibodies, one male New Zealand white rabbit was administered approximately 15 μg of purified M_{II}-PST intradermally in complete Freund's adjuvant at 30 sites along the back, as described by Vaitukaitis *et al.* (10). Six weeks later, the rabbit received a booster injection containing an additional 15 μg of purified M_{II}-PST in Freund's incomplete adjuvant. After 10 days, the rabbit was test bled and the serum was evaluated for the presence of specific antibodies to PST by immunoblot analysis.

Immunoblot analysis. The 100,000 × *g* supernatant solutions of various human and nonhuman tissues were subjected to sodium doedcyl sulfate-polyacrylamide gel electrophoresis using the method of Laemmli (11) in a 12% polyacrylamide gel. Proteins were electrophoretically transferred from the gel to nitrocellulose paper (12), using a Bio-Rad transblot apparatus. The nitrocellulose was blocked with 3% gelatin in 100 mM Tris, pH 7.5, that contained 500 mM NaCl (TBS), for 1 hr. Incubation with both the primary and secondary antibodies was performed in TBS that contained 0.1% Tween 20 and 1% gelatin, at room temperature. Primary antibody was routinely utilized at a dilution of 1/10,000 and occasionally at a 1/1,000 dilution, where noted. The nitrocellulose was incubated overnight with primary antibody and then for 1 hr with secondary antibody conjugated with alkaline phosphatase. Washes between incubation steps were in TBS that contained 0.1% Tween 20, for 15 min at room temperature. An alkaline phosphatase substrate kit (Vector Laboratories, Inc., Burlingame, CA) was used to develop the immunoblots.

Other methods. Protein concentrations were determined by the procedure of Bradford (13).

Results

Immunoblot analysis of a 100,000 × *g* supernatant of human platelets, using the post-boost serum of a rabbit immunized with human platelet M_{II}-PST, indicated the presence of antibodies directed against 34- and 32-kDa polypeptides (Fig. 1). The immunostained band observed at 34 kDa is consistent with the subunit molecular mass of M_{II}-PST, whereas the band at 32 kDa most likely represents a different form of PST that cross-reacts with this antibody preparation. To further investigate the relationship of these immunoreactive polypeptides to platelet M- and P-PST activity, fractions eluting from a DEAE-cellulose column to which the platelet supernatant solution had been applied were both assayed for M- and P-PST activity and

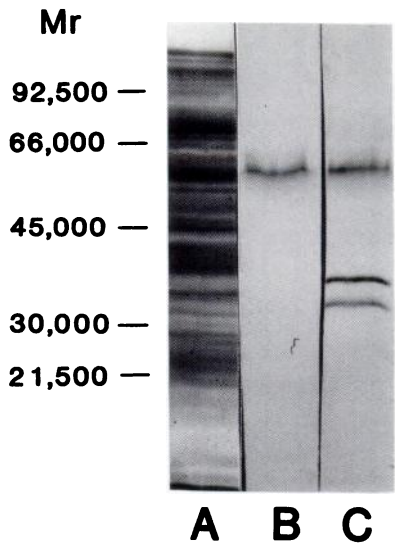

Fig. 1. Immunoblot analysis of a 100,000 × *g* human platelet supernatant solution. Approximately 40 μg of protein were applied to each lane. *Lane A* was stained for protein with Coomassie blue, whereas *lanes B* and *C* were immunoprobed with a 1/10,000 dilution of preimmune and immune serum, respectively.

immunoprobed with the anti-M_{II}-PST serum. As illustrated in Fig. 2A, two peaks of phenol-sulfating activity, previously defined as P_I- and P_{II}-PST (7), and one peak of dopamine-sulfating activity can be seen following the elution of the anion exchange column with a linear salt gradient. Immunoblot analysis of individual fractions (Fig. 2C) demonstrated the initial appearance of the 32-kDa polypeptide eluting from the column, which was followed by the appearance of the 34-kDa polypeptide. Results from quantitative densitometric scanning (LKB Ultrascan XL Enhanced Laser Densitometer), illustrated in Fig. 2B, reveal a coincidence between the concentration of the 32-kDa species and the two peaks of P-PST activity. Furthermore, the concentration of the 34-kDa protein was found to closely correspond to the elution of M-PST activity.

To determine the distribution of both M- and P-PST in humans, various tissues were immunoprobed with a 1/10,000 dilution of anti-PST serum, as shown in Fig. 3. Liver (Fig. 3, *lane D*) and adrenal gland (Fig. 3, *lane F*) contained a prominent immunoreactive 32-kDa band, with no detectable 34-kDa species. The 32-kDa band in these tissues comigrated with the 32-kDa band (P-PST) in platelets (Fig. 3, *lane A*). Quantitative densitometry (Table 1) indicated that adrenal gland contained about a 2-fold greater level of the 32-kDa polypeptide than did liver. Although both immunoreactive polypeptides were detectable in human placenta (Fig. 3, *lane G*), the 34-kDa polypeptide was present in a 3-fold greater abundance. Under the conditions employed, immunoreactive protein species were not observed using preimmune serum, except when human platelets were analyzed (Fig. 1). In contrast to the tissues described above, immunoblots of human lung, kidney, jejunum, frontal cortex, and pituitary gland (Fig. 3, *lanes B, C, E, H,* and *I*, respectively) showed no detectable 32- or 34-kDa bands, using a 1/10,000 dilution of primary antiserum. No change in these results was observed when the concentration of primary antiserum was increased to 1/1,000 dilution.

To further determine whether human brain P- and M-PST are capable of cross-reacting with the antibody raised against platelet PST, immunoblots were performed with human brain

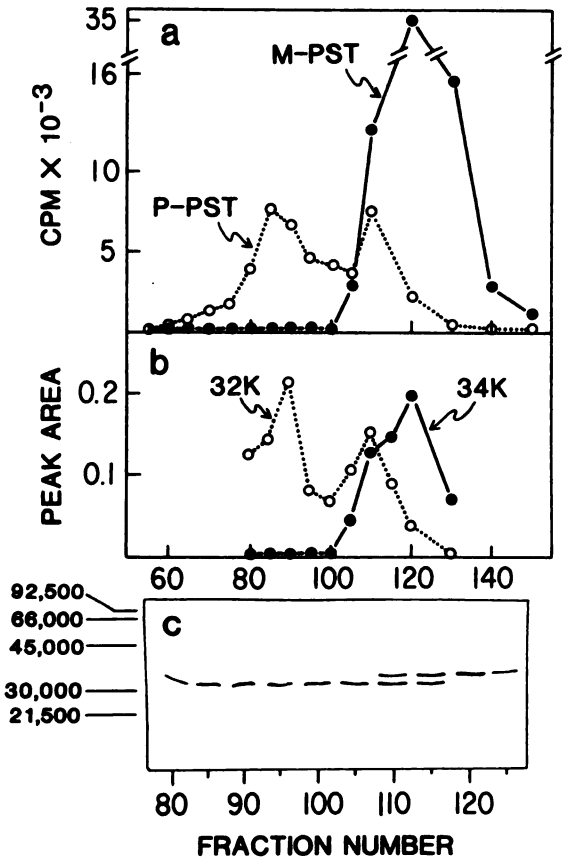

Fig. 2. Measurement of M- and P-PST activity and immunoblot analysis of fractions obtained from DEAE-cellulose chromatography of human platelet 100,000 × *g* supernatant solution. A, Dopamine- (●) and phenol-sulfating (○) activity. B, Densitometric analysis of the immunoblot obtained from C. C, Immunoblot of the fractions using a 1/10,000 dilution of primary M_H-PST antiserum.

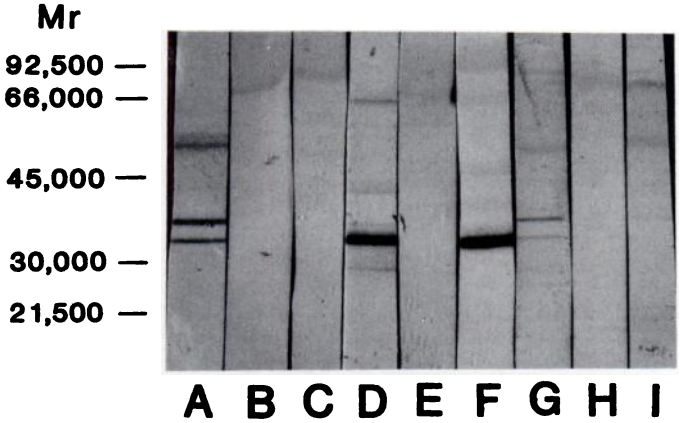

Fig. 3. Immunoblot analysis of human tissue supernatant solutions. Approximate quantities of protein applied to lanes is as follows: *lane A* (platelets), 40 μg; *lane B* (lung), 103 μg; *lane C* (kidney), 56 μg; *lane D* (liver), 30 μg; *lane E* (jejunum), 46 μg; *lane F* (adrenal gland), 16 μg; *lane G* (placenta), 85 μg; *lane H* (frontal cortex), 32 μg; *lane I* (pituitary gland), 94 μg. Each *lane* was probed with a 1/10,000 dilution of primary antiserum.

PST that was partially purified by anion exchange chromatography. As illustrated in Fig. 4, fractions from the column that contained both P- and M-PST activity displayed both a 32- and a 34-kDa band on the immunoblot, at a 1/1000 dilution of antisera.

TABLE 1

Immunoreactivity of M- and P-PST from a variety of human tissues as determined by quantitative densitometry

Tissue	Immunoreactivity[a]	
	M-PST	P-PST
Adrenal gland	ND[b]	68.4
Liver	ND	33.1
Placenta	2.2	0.78
Platelets	11.0	8.9
Kidney	ND	ND
Jejunum	ND	ND
Lung	ND	ND
Frontal cortex	ND	ND
Pituitary gland	ND	ND

[a] Immunoreactivity is defined as the scanned peak area of the 32 or 34 kDa band from the immunoblots (Fig. 3) per μg of protein × 1000.
[b] ND, not detectable.

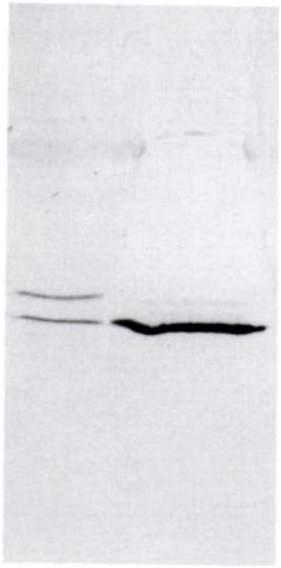

Fig. 4. Immunoblot analysis of partially purified human brain P- and M-PST (A) and purified human liver P-PST (B). Concentration of protein applied to each lane was 60 μg (A) and 0.16 μg (B). Each *lane* was probed with a 1/1000 dilution of primary antiserum.

We next examined several fresh human livers to determine whether there was a relationship between the specific activity of phenol sulfation and the tissue PST content based on immunoblot analysis. As indicated by the data in Fig. 5, a strong correlation ($r = 0.94$) between the peak area of the 32-kDa immunostained band and the specific activity of P-PST from four liver samples was observed.

In addition, we examined the ability of the antibodies raised against human M_{II}-PST to cross-react with PST from various animal livers. Fig. 6 illustrates the immunoblot of liver samples from several animal species. Although several immunoreactive bands were observed for each of the samples, the most immunoreactive proteins were of an approximate molecular mass as follows: monkey (Fig. 6, *lane A*); 32 and 32.5 kDa; mouse (Fig. 6; *lane B*); 32 kDa; guinea pig (Fig. 6, *lane C*); 32.5 kDa; rat (Fig. 6, *lane D*); 32 and 33 kDa; and frog (Fig. 6, *lane E*); 33 and 34 kDa. These immunoreactive polypeptides were not present when control serum was utilized (data not shown).

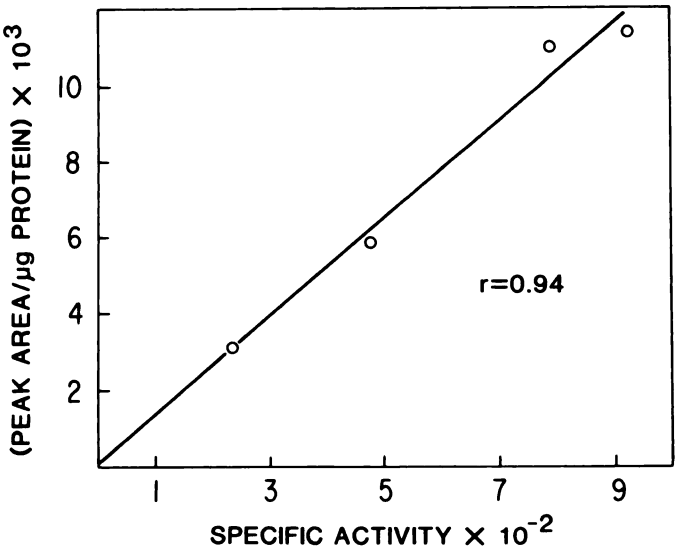

Fig. 5. Quantitation of P-PST in human liver samples. The 100,000 × *g* supernatant solutions of liver samples from four individuals were analyzed with a 1/10,000 dilution of primary antiserum. Varying amounts of protein from each liver sample were utilized in duplicate for immunoblot analysis. For quantitation of the 32-kDa immunoreactive polypeptide, immunoblots were scanned twice using a LKB Ultroscan XL scanning densitometer.

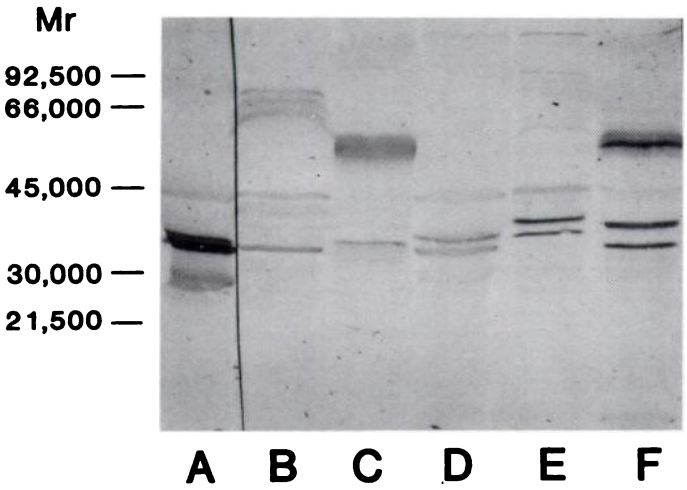

Fig. 6. Immunoblot analysis of liver samples from various animals. The 100,000 × *g* supernatant solutions of various nonhuman livers were probed with a 1/10,000 dilution of anti-human PST serum. The approximate amounts of protein applied to each lane were as follows: *lane A* (monkey), 89 μg; *lane B* (mouse), 183 μg; *lane C* (guinea pig), 93 μg; *lane D* (rat), 68 μg; *lane E* (frog), 78 μg. A human platelet sample (*lane F*, approximately 40 μg of protein) was included as a human M-PST and P-PST reference standard.

Discussion

We report here for the first time the immunological identification of multiple forms of PST in human tissues. As indicated in Fig. 1, antibodies raised against M_{II}-PST not only reacted with a 34-kDa polypeptide, previously identified as M_{II}-PST (8), but also intensely cross-reacted with a 32-kDa polypeptide. Based on the activity and the immunostaining pattern of the platelet 100,000 × *g* supernatant solution fractions obtained from the DEAE-cellulose column (Fig. 2), it can be seen that the 34- and the 32-kDa bands correspond to the elution profile of dopamine- and phenol-sulfating activities, respectively. These data imply that the immunoreactive 32-kDa polypeptide represents the subunit of P-PST. This is further substantiated by the fact that the concentration of the 32-kDa immunoreactive polypeptide observed in four separate livers (Fig. 4) significantly correlates with their respective P-PST activity. In addition, we have recently purified human liver P-PST to homogeneity and have found the enzyme to have a native and subunit molecular weight of approximately 66 and 32 kDa, respectively.[1] Therefore, our data are consistent with M- and P-PST existing as homodimers *in vivo*, with subunits of 34 kDa for M-PST and 32 kDa for P-PST.

Evaluation of a variety of human tissues by immunoblot analysis (Fig. 3; Table 1) revealed that liver and adrenal gland contain the most abundant levels of P-PST, suggesting that these tissues possess the highest capacity to sulfoconjugate phenolic xenobiotics, including many therapeutically relevant drugs, in humans. In addition, placenta was found to contain immunologically detectable levels of both M- and P-PST. The presence of PST in placenta may function to protect the fetus from unacceptably high levels of phenolic compounds and monoamines present in the maternal bloodstream. Although PST was immunologically undetectable in lung, kidney, frontal cortex, pituitary gland, and jejunum supernatants utilizing secondary antibodies conjugated with alkaline phosphatase, use of the more sensitive biotin-avidin detection system and a 10-fold higher concentration of primary antibody did allow us to visualize PST on immunoblots of several of these tissues. For example, we have observed a faint 34-kDa band on immunoblots of both frontal cortex and kidney and a 32-kDa band on immunoblots of lung (data not shown). However, under conditions employed with the biotin-avidin system, nonspecific staining was consistently a significant problem when either immune or preimmune serum was used, thus preventing us from routinely employing this more sensitive method for detecting PST in tissues. As shown in Fig. 4, when human brain P- and M-PST were partially purified, we were indeed able to see the 32- and 34-kDa bands on immunoblots with alkaline phosphatase. Our observation of low amounts of immunodetectable PST in lung, frontal cortex, and pituitary gland is substantiated by published reports of low PST activity in these tissues (7, 14, 15). Rein and co-workers (4) have reported a high specific activity for M-PST in human jejunum, a level of enzyme that should have been easily visualized with our immunodetection system. It is possible that significant hydrolysis of PST occurred in our jejunum samples obtained at the time of autopsy, because the intestine is known to contain high concentrations of proteolytic enzymes (16). This hypothesis is supported by the lack of measureable PST activity present in autopsied samples of this tissue measured in our laboratory. It should also be pointed out that the data in Table 1, comparing the relative abundance of M- and P-PST in the different tissues, may be subject to some limitations because it is possible that the color yield may not be the same for the two proteins.

There is a minimum 5-fold variation in PST activity between individuals (17). Consistent with this is our observation that P-PST specific activity measured in four different liver samples was found to vary as much as 4-fold. Therefore, it was of interest to evaluate the relationship between the amount of

[1] J. A. Heroux and J. A. Roth, unpublished data.

immunoreactive PST and the activity of PST from livers of various individuals. The strong correlation between phenol-sulfating activity and the peak area of the immunoreactive 32-kDa polypeptide, as determined by quantitative densitometry (Fig. 4), suggests that variations in P-PST specific activity between people are not due to differences in the biochemical properties of PST or to the presence of endogenous inhibitors or activators but most likely result from differences in the tissue concentration of the enzyme.

PST activity has been previously identified in several animal species including the rat (18), guinea pig (19), and dog (20). These studies have provided an abundance of information regarding the biochemical properties of mammalian PST and have highlighted differences between human and nonhuman forms of the enzyme (3, 8). Interestingly, in the present report, anti-human M_{II}-PST cross-reacted with a variety of proteins from nonhuman livers including a 32-kDa polypeptide in the monkey, mouse, and rat, a 32.5-kDa polypeptide in the monkey and guinea pig, and a 33-kDa polypeptide in the rat and frog (Fig. 5). We have identified one of the immunoreactive proteins in rat liver as aryl sulfotransferase IV, using two lines of evidence. Firstly, our anti-human PST serum significantly cross-reacted with purified rat liver aryl sulfotransferase IV. Secondly, the observed immunoreactive 33-kDa protein in rat liver (Fig. 6, *lane D*) possessed a mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis identical to that of purified rat liver aryl sulfotransferase IV. Although the identity of the immunoreactive proteins of 32 to 34 kDa from the other animal livers (Fig. 5, *lanes A, B, C,* and *E*) cannot be determined at this time, it is interesting to speculate that they are other forms of PST. If this is the case, considerable homology in primary amino acid sequence may exist between the human and nonhuman PST enzymes.

In conclusion, on the basis of immunological techniques, we have presented the first evidence that the subunit molecular weight of human P-PST is approximately 32 kDa and differs significantly from that of M-PST. In addition, different amounts of M- and P-PST were demonstrated in liver, adrenal gland, placenta, and platelets, whereas no immunologically detectable levels of either PST form were observed in other tissues, indicating that human PST is under strict tissue-specific regulation.

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References

1. Roy, A. B. in *Sulfation of Drugs and Related Compounds* (G. J. Mulder, ed.). CRC Press, Boca Raton, FL 83–99 (1981).
2. Johnson, C. A., C. A. Baker, and R. T. Smith. Radioenzymatic assay of sulfate conjugates of catecholamines and DOPA in plasma. *Life Sci.* **26**:1591–1598 (1980).
3. Roth, J. A. Phenol sulfotransferase, in *Neuromethods, Vol. 5, Neurotransmitter Enzymes* (A. A. Boulton, G. B. Baker, and P. H. Yu, eds.). Humana Press, Clifton, NJ, 575–604 (1986).
4. Rein, G., V. Glover, and M. Sandler. Evidence for multiple forms of human phenol sulfotransferase. *Psychopharmacol. Bull.* **17**:43–45 (1981).
5. Reiter, C., G. Mwaluko, J. Dunnette, J. Van Loon, and R. Weinshilboum. Thermolabile and thermostable human platelet phenol sulfotransferase: substrate specificity and physical separation. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **324**:140–147 (1983).
6. Rein, G., V. Glover, and M. Sandler. Multiple forms of phenolsulphotransferase in human tissues: selective inhibition by dichloronitrophenol. *Biochem. Pharmacol.* **31**:1893–1897 (1982).
7. Whittemore, R. M., and J. A. Roth. Purification and kinetic characterization of a dopamine-sulfating form of phenol sulfotransferase from human brain. *Biochemistry* **24**:2477–2482 (1986).
8. Heroux, J. A., and J. A. Roth. Physical characterization of a monoamine-sulfating form of phenol sulfotransferase from human platelets. *Mol. Pharmacol.* **34**:194–199 (1988).
9. Whittemore, R. M., and J. A. Roth. A modified ecteola cellulose assay for M and P phenol sulfotransferase. *Biochem. Pharmacol.* **43**:1647–1652 (1985).
10. Vaitukaitus, J., J. B. Robbins, E. Nieschlag, and G. T. Ross. A method for producing specific antisera with small doses of immunogen. *J. Clin. Endocrinol. Metab.* **33**:988–991 (1971).
11. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
12. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
13. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
14. Baranczyk-Kuzma, A. Phenol sulfotransferase in human lung. *Biochem. Med. Metab. Biol.* **35**:18–30 (1986).
15. Anderson, R. J., J. K. Yoon, E. G. Sinsheimer, and B. L. Jackson. Human pituitary phenol sulfotransferase: biochemical properties and activities of the thermostable and thermolabile forms. *Neuroendocrinology* **44**:117–124 (1986).
16. White, A., P. Handler, E. L. Smith, R. L. Hill, and I. R. Lehman. *Principles of Biochemistry.* McGraw-Hill Book Co., New York, 674–680 (1978).
17. Reiter, C., and R. M. Weinshilboum. Acetaminophen and phenol: substrates for both a thermostable and thermolabile form of human platelet phenol sulfotransferse. *J. Pharmacol. Exp. Ther.* **221**:43–51 (1982).
18. Sekura, R. D., and W. B. Jacoby. Phenol sulfotransferase. *J. Biol. chem.* **254**:5658–5663 (1979).
19. Banerjee, R. K., and A. B. Roy. The sulfotransferases of guinea pig liver. *Mol. Pharmacol.* **2**:56–66 (1966).
20. Romain, Y., S. Demassieux, and S. Carriere. Partial purification and characterization of two isozymes involved in the sulfurylation of catecholamines. *Biochem. Biophys. Res. Commun.* **106**:999–1005 (1982).

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