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Substrate Specificity of Human Monoamine (M)-Form Phenol Sulfotransferase: Preparation and Analysis of Dopa 3-*O*-Sulfate and Dopa 4-*O*-Sulfate[1]

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Upon two-dimensional thin-layer separation, the sulfated L-3,4-dihydroxyphenylalanine (L-DopaS) generated enzymatically was found to co-migrate with only one of the two ninhydrin-stained spots corresponding to the two sulfated forms (3-*O*-sulfate and 4-*O*-sulfate) of synthetic L-DopaS. To clarify precisely the identity of the enzymatically generated L-DopaS, the two sulfated forms of synthetic L-DopaS were separated and purified using high performance liquid chromatography. Purified L-Dopa 3-*O*-sulfate and L-Dopa 4-*O*-sulfate were identified by ^{1}H-nuclear magnetic resonance (NMR) spectrometry and used as standards in the analysis of the L-DopaS generated during metabolic labeling of HepG2 human hepatoma cells or enzymatic assay using recombinant human monoamine (M)-form phenol sulfotransferase. The results obtained demonstrated unequivocally the generation of L-Dopa 3-*O*-sulfate, indicating the specificity of the M-form phenol sulfotransferase being for the meta-hydroxyl group of L-Dopa.

Key words: Dopa, high performance liquid chromatography, human M-form phenol sulfotransferase, nuclear magnetic resonance spectrometry, substrate specificity.

In mammals, enzymatic sulfation plays a variety of functional roles including modulation of the biological activities of peptides or proteins (*1*), detoxification and excretion of drugs and xenobiotics (*2, 3*), and regulation of the metabolism of thyroid hormones and catecholamines (*4*). In relation to the catecholamine metabolism, we have recently demonstrated, following a 40-year controversy (*2*), the occurrence of the sulfation of free amino acid form of L-papa (p)-tyrosine in mammalian cells (*5-7*). A somewhat puzzling issue regarding this finding, however, is: why should mammalian cells carry out the sulfation of an amino acid which is needed for protein synthesis? To convert L-p-tyrosine to L-p-tyrosine *O*-sulfate (L-p-TyrS), a compound destined for excretion (*8*), would seem to be counterproductive in terms of cellular economy. The question that then ensues is: does L-p-tyrosine truly represent the physiological substrate of the enzyme responsible for the sulfation reaction?

Using HepG2 human hepatoma cells as a model, we have demonstrated in a recent study that other tyrosine derivatives/isomers, *e.g.*, L-Dopa and L-meta (m)-tyrosine, are in fact better substrates for sulfation than is L-p-tyrosine (*9*). Furthermore, upon two-dimensional thin-layer separation, the radioactive L-Dopa[^{35}S], generated during the enzymatic assay using 3′-phosphoadenosine 5′-phospho[^{35}S]-sulfate (PAP[^{35}S]) as the sulfate donor, co-migrated with only one of the two ninhydrin-stained spots presumably corresponding to the two sulfated forms (3-*O*-sulfate and 4-*O*-sulfate) of synthetic L-DopaS. These findings imply a substrate specificity of the HepG2 Dopa/tyrosine-sulfating enzyme with respect to the spatial or geometric positioning of the aryl hydroxyl group involved in the sulfation reaction. Preliminary attempts to identify the enzyme revealed that the Dopa/tyrosine-sulfating activities present in HepG2 cell homogenate, upon DEAE-Bio-Gel and hydroxyapatite column chromatography, co-eluted with that of the M-form phenol sulfotransferase (*9*). By employing the reverse transcriptase-polymerase chain reaction technique; we have recently cloned the human M-form phenol sulfotransferase and demonstrated the recombinant enzyme expressed in COS-7 cells to display Dopa/tyrosine-sulfating activities (*10*).

We report here the preparation and ^{1}H-NMR analysis of the two sulfated forms of L-DopaS generated *via* chemical synthesis and their use in the identification of the sulfated L-Dopa generated during the M-form phenol sulfotransferase-catalyzed sulfation reactions.

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Abbreviations: Dopa, 3,4-dihydroxyphenylalanine; TyrS, tyrosine *O*-sulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance spectrometry.

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MATERIALS AND METHODS

Materials—L-Dopa, *m*-hydroxybenzylhydrazine, ninhydrin, aprotinin, antipain, benzamidine, soybean trypsin inhibitor, dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), adenosine 5′-triphosphate (ATP), adenosine 5′-monophosphate (5′-AMP), *N*-2-hydroxylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), 3-[dimethyl(hydroxylmethyl)-methylamino]-2-hydroxypropanesulfonic acid (Ampso), minimum essential medium (MEM), fetal bovine serum, penicillin G, and streptomycin sulfate were products of Sigma Chemical. A mixture of L-Dopa 3-*O*-sulfate and L-Dopa 4-*O*-sulfate (collectively abbreviated as L-DopaS) was synthesized according to the procedure of Jevons (*11*). Carrier-free sodium [^{35}S]sulfate was from ICN Biomedicals. PAP[^{35}S] (15 Ci/mmol) was synthesized from ATP and [^{35}S]sulfate using the sulfate-activating enzymes, ATP sulfurylase and adenosine 5′-phosphosulfate kinase, from *Bacillus stearothermophilus* as described previously (*12*). Chromatogram cellulose thin-layer chromatography (TLC) plates were products of Eastman Kodak. HepG2 human hepatoma cells (ATCC HB 8065) were from American Type Culture Collection. Recombinant human M-form phenol sulfotransferase expressed in *Escherichia coli* was prepared and purified based on the procedure described previously (*13*). All other chemicals were of the highest grades commercially available.

Cell Culture—HepG2 human hepatoma cells were maintained, under a 5% CO_2 atmosphere at 37°C, in MEM supplemented with 10% fetal bovine serum, penicillin G (30 μg/ml), and streptomycin sulfate (50 μg/ml). Confluent cells, grown in 100-mm tissue culture dishes or in individual wells of 24-well culture plates, were used in the following experiments.

Preparation of the HepG2 Cell Homogenate—Confluent HepG2 cells, grown in 15 100-mm dishes, were scraped off, suspended in 10 ml of 20 mM Hepes-NaOH (pH 7.0) containing 250 mM sucrose, 30 μg/ml aprotinin, and 1 mM PMSF, and homogenized using a Dounce tissue grinder. The crude homogenate was centrifuged at 140,000 × *g* for 2 h at 4°C. The supernatant containing the cytosolic proteins was dialyzed at 4°C overnight against 10 mM Hepes-NaOH (pH 7.0) plus 250 mM sucrose. The dialyzed fraction was used in the enzymatic assay.

Metabolic Labeling of HepG2 Cells with [^{35}S]Sulfate in the Presence of L-Dopa—Confluent HepG2 cells grown in individual wells of a 24-well culture plate, pre-incubated in sulfate-free (prepared by omitting streptomycin sulfate and replacing magnesium sulfate with magnesium chloride), phenylalanine- and tyrosine-deficient MEM for 12 h, were labeled with 0.25-ml aliquots of the same medium containing [^{35}S]sulfate (0.3 mCi/ml), 1 mM L-Dopa, and 0.1 mM *m*-hydroxybenzylhydrazine, an aromatic amino acid decarboxylase inhibitor (*14*). At the end of a 20-h labeling, the media were collected, spin-filtered, and subjected to the analysis of L-Dopa[^{35}S] using a two-dimensional thin-layer separation procedure previously developed (*15*).

Enzymatic Assay—HepG2 homogenate or purified recombinant M-form phenol sulfotransferase was assayed for Dopa-sulfating activity using PAP[^{35}S] as the sulfate donor. The assay mixture, with a final volume of 50 μl, contained 50 mM Ampso-NaOH (pH 8.75), 250 mM sucrose, 25 mM NaF, 1 mM 5′-AMP, protease inhibitors (30 μg/ml aprotinin, 30 μg/ml antipain, 300 μg/ml benzamidine, and 30 μg/ml soybean trypsin inhibitor), 14 μM PAP-[^{35}S] (4.4 Ci/mmol), and 1 mM L-Dopa. The reaction was started by the addition of the enzyme preparation, allowed to proceed for 30 min at 37°C, and terminated by heating at 100°C for 3 min. The precipitates formed were cleared by centrifugation. The clear supernatant was analyzed for the L-Dopa[^{35}S] produced based on a two-dimensional thin-layer separation procedure previously developed (*15*).

High Performance Liquid Chromatography—The reversed-phase and chiral high-performance liquid chromatography (HPLC) were carried out using a CCPN-II solvent delivery module (Tosoh) coupled to a UV-8020 detector. A reversed-phase column (250 mm × 4.6 mm I.D.) packed with Asahipack ODP-504E (5 mm) (Showa Denko) and a chiral separation column (150 mm × 0.4 mm I.D.) packed with CROWNPAK CR(+) (Daicel Chemical) were used. UV absorbance of compounds eluted from the column was measured at 280 nm wavelength. All samples were injected *via* a 100-ml loop and a rotary valve (Tosoh). To separate the two sulfated forms of synthetic L-Dopa-sulfate, the mixture of L-Dopa-3-sulfate and L-Dopa-4-sulfate was applied onto the chiral column and eluted at 20°C with a solution of 0.098% (v/v; pH 2.0) of perchloric acid in HPLC-grade water at a flow rate of 0.4 ml/min. Fractions corresponding to two UV-absorbing peaks, Peak 1 and Peak 2 (*cf.* "RESULTS AND DISCUSSION"), were collected, neutralized with 1 N KOH, and lyophilized. The residues were dissolved in water. The dissolved samples were applied onto the reversed-phase HPLC column and eluted at 40°C with HPLC-grade water at a flow rate of 0.8 ml/min.

^{1}H-NMR Spectrometry—^{1}H-NMR spectra were recorded at 90 MHz using a JEOL EX-90 Spectrophotometer. Samples (1.0-10 mg) were dissolved in 1 N NaOD in deuterium oxide (100-300 μl, 99.99D%). The chemical shifts were presented in ppm with dioxane as an internal standard (δ = 3.56 ppm). The residual HDO signal was quenched using the homogate decoupling method. In measuring samples containing Dopa, the solvent was treated in an ultrasonic bath for 10 min and dry nitrogen was bubbled through the solvent for 5 min to remove the dissolved oxygen.

RESULTS AND DISCUSSION

As shown in Fig. 1, upon two-dimensional thin-layer separation, the L-Dopa[^{35}S] generated during the enzymatic sulfation of L-Dopa catalyzed by the HepG2 enzyme comigrated with one of the two ninhydrin-positive species (presumably corresponding to Dopa 3-*O*-sulfate and Dopa 4-*O*-sulfate) of the synthetic L-DopaS. These results indicate that the enzymatic sulfation of L-Dopa occurs specifically on only one of the two ring hydroxyl groups. To clarify the identity of the L-Dopa[^{35}S] as 3-*O*-sulfate or 4-*O*-sulfate, we decided to separate these two sulfated forms and use them as standards for identification.

HPLC Separation and ^{1}H-NMR Spectrometry of L-Dopa 3-O-Sulfate and L-Dopa 4-O-Sulfate—To separate the two sulfated forms of synthetic L-DopaS, HPLC was employed. HPLC using a chiral separation column yielded two distinct peaks (Fig. 2). The sulfated L-Dopa forms collected in these

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two peaks (designated Peak 1 and Peak 2) were further purified using a reversed-phase column. The purified compounds were analyzed using ^{1}H-NMR spectrometry with respect to their identities as L-Dopa 3-*O*-sulfate or L-Dopa 4-*O*-sulfate. Figure 3 shows the NMR spectra measured for the compounds eluted in Peak 1 and Peak 2, respectively. The chemical shifts were assigned to the methylene protons and aromatic protons (*cf.* Fig. 4) by comparison with those measured for L-Dopa and sulfated and unsulfated forms of L-p-tyrosine and L-m-tyrosine under the same conditions.

The methylene protons adjacent to a chiral center are known to be nonequivalent (*16*). In the case of L-p-tyrosine, the chemical shifts of the two methylene protons were determined to be 2.44 and 2.69 ppm, respectively. Each of the two protons was split equally by the other ($J_{gem}=14$ Hz) and unequally by the vicinal proton ($J_{vic}=5.3$ and 7.8 Hz), resulting in eight peaks (figure not shown). The δ value ranges of the methylene protons of L-p-tyrosine, L-p-TyrS, L-m-tyrosine, L-m-TyrS, and Peak 1 and Peak 2 compounds are compiled in Table I. The methylene protons of L-p-tyrosine and L-m-tyrosine resonanced in the 2.3–2.9 ppm range, indicating that the position (para or meta) of the hydroxyl group on the aromatic ring has little effect on the chemical shift of the methylene protons. When the hydroxyl group was sulfated, the methylene protons of L-p-tyrosine displayed a downfield shift of about 0.5 ppm, whereas the methylene protons of L-m-tyrosine were little affected.

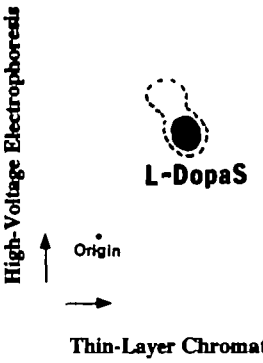

Fig. 1. **Enzymatic sulfation of L-Dopa using PAP[^{35}S] as the sulfate donor.** The protein concentration of the HepG2 cytosolic fraction used in the enzymatic assay was 0.54 mg/ml. The figure shows the autoradiograph taken from the TLC plate used for the two-dimensional thin-layer analysis of the reaction mixture. The dashed line circle indicates the positions of carrier synthetic L-DopaS detected by ninhydrin staining.

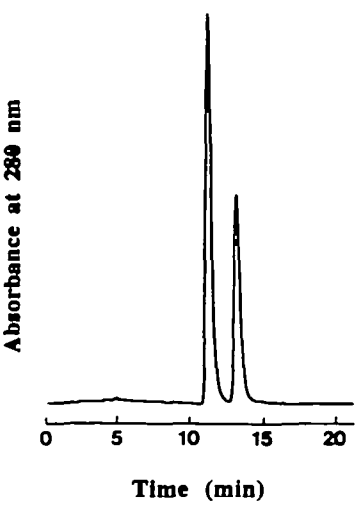

Fig. 2. **Chiral HPLC spectrum of synthetic L-DopaS.** The two forms of synthetic L-DopaS eluted from the chiral column were monitored based on absorbance at 280 nm wavelength.

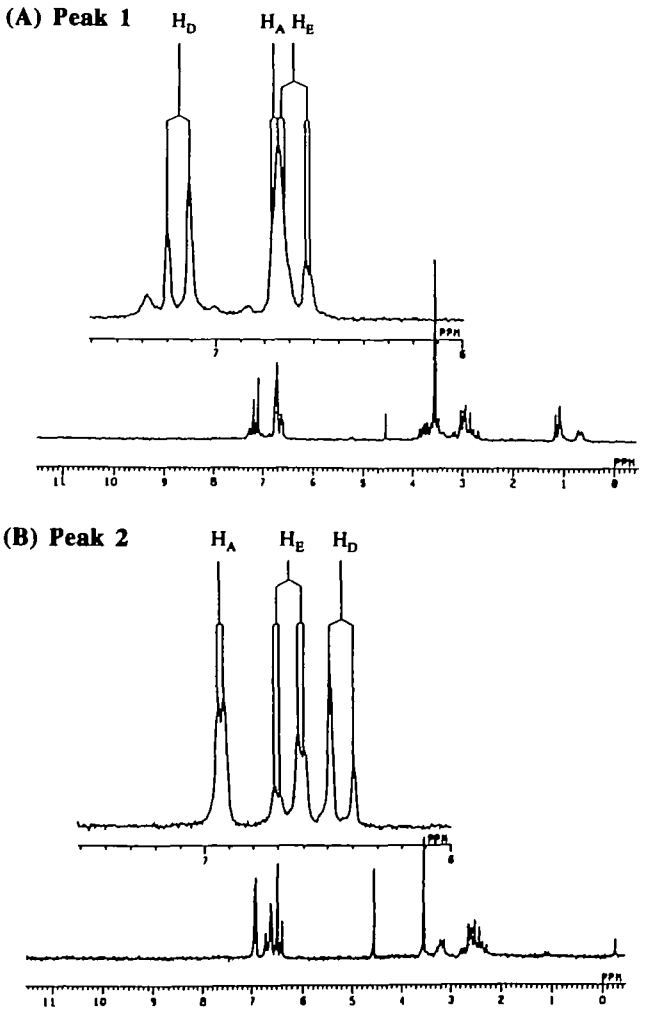

Fig. 3. **^{1}H-NMR spectra of the two forms of L-DopaS eluted in Peak 1 and Peak 2 upon chiral HPLC.** Parts (A) and (B) correspond to the spectra taken for the sulfated L-Dopa eluted in Peak 1 and Peak 2, respectively.

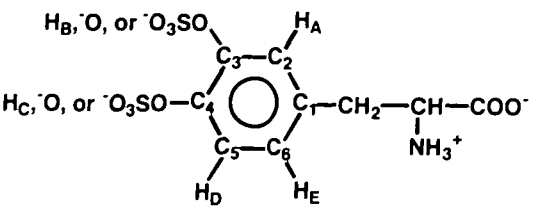

Fig. 4. **A general chemical structure showing the methylene and aromatic protons of L-p-tyrosine, L-m-tyrosine, L-Dopa, and their sulfate conjugates.**

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TABLE I. **Chemical shifts of methylene and aromatic protons of L-p-tyrosine, L-m-tyrosine, L-Dopa, and their sulfate conjugates.**

Protons	Chemical shift (in ppm)						
	L-p-Tyr	L-p-Tyr-S	L-m-Tyr	L-m-TyrS	L-Dopa	Peak 1	Peak 2
$-CH_2-$	2.3-2.9	2.8-3.4	2.3-2.9	2.4-3.0	2.2-2.8	2.7-3.2	2.3-2.9
H_A	6.80	7.13	6.30	6.9-7.3	6.21	6.72	6.93
H_B	6.45	7.13	—	—	—	—	—
H_C	—	—	6.30	6.9-7.3	—	—	—
H_D	6.37	7.13	6.92	6.9-7.3	6.28	7.13	6.45
H_E	6.80	7.13	6.30	6.9-7.3	6.10	6.65	6.65

By comparing the chemical shifts determined for the methylene protons of the two sulfated forms of L-Dopa with those determined for the methylene protons of L-p-TyrS and L-m-TyrS, it can be concluded that the compounds eluted in Peak 1 and Peak 2 are most likely L-Dopa 4-sulfate and L-Dopa 3-sulfate, respectively.

Analysis of the NMR spectra of aromatic protons also provided support for the above assignment. L-p-Tyrosine exhibited a typical coupling pattern for an AA'XX' system, much like the AX system (*16*). As shown in Table I, the meta protons (H_A and H_E, $\delta = 6.80$) to the hydroxyl group in L-p-tyrosine were more deshielded by the hydroxyl group than the ortho protons (H_B and H_D, $\delta = 6.45$ and 6.37). When the hydroxyl group was sulfated, only a single broad peak at 7.13 ppm was measured for aromatic protons (figure not shown), indicating that the two inner peaks of the doublets had merged and the outer peaks had disappeared. This is probably due to the similar chemical shifts of the ortho and meta protons. Sulfation of the hydroxyl group results in the reduction of electron-donating capacity of the hydroxyl group and the large downfield shift of particularly the ortho protons. The same tendency was also observed for L-m-TyrS, with a single broad peak spanning 6.9-7.3 ppm. In comparison with the chemical shifts determined for L-Dopa, the sulfated L-Dopa eluted in Peak 1 displayed a large downfield shift for H_D (assumed to be at ortho position to the sulfated hydroxyl group), and the sulfated L-Dopa eluted in Peak 2 displayed a large downfield shift for H_A (assumed to be at ortho position to the sulfated hydroxyl group). The two forms of L-DopaS eluted in Peak 1 and Peak 2 therefore resembled, respectively, L-p-TyrS and L-m-TyrS in manifesting the effect of sulfation.

When the data on the chemical shifts of aromatic protons are examined from a different aspect, some 1,2,4-trisubstituted benzene derivatives show a typical coupling pattern for their aromatic protons: H_A a doublet, H_D a doublet, and H_E a double-doublet (*16*). Ordinarily, the ortho coupling constant (J_{DE}) and the meta coupling constant (J_{AE}) are 6-10 and 1-3 Hz, and the para coupling constant is nearly 0 Hz. The aromatic protons of L-Dopa and the two forms of L-DopaS (Peak 1 and Peak 2) displayed the typical coupling pattern for the 1,2,4-trisubstituted benzene derivatives (*cf.* Fig. 4). Their aromatic protons can thus be assigned unequivocally based on the data compiled in Table I. In the spectrum taken for L-Dopa, H_A, H_D, and H_E resonanced, respectively at 6.21, 6.28, and 6.10 ppm. Upon sulfation of the hydroxyl group on C_3 (for the sulfated L-Dopa eluted in Peak 2), H_A shifted considerably to downfield. Sulfation of the hydroxyl group on C_4 (for the sulfated L-Dopa eluted in Peak 1), on the other hand, resulted in a large downfield shift of H_D. By combining these results with those presented above, it can be concluded that the two sulfated forms of L-Dopa eluted in Peak 1 and Peak 2 during the HPLC performed are L-Dopa 4-sulfate and L-Dopa 3-sulfate, respectively.

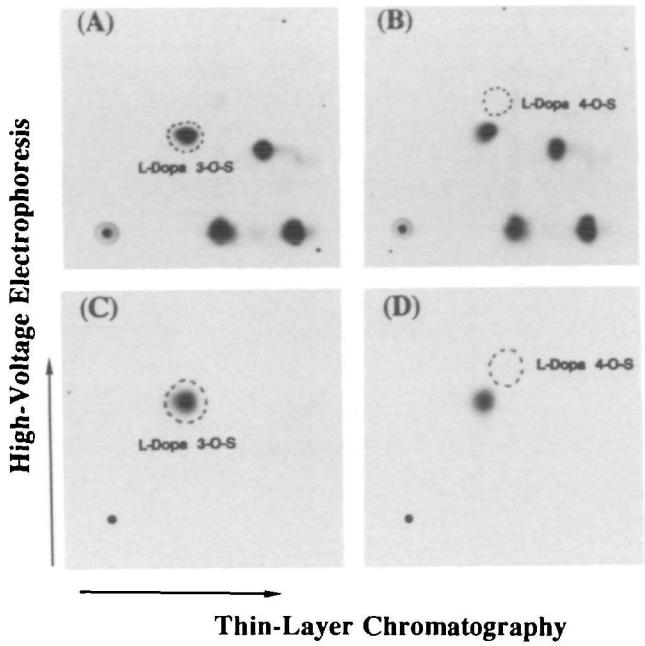

Fig. 5. **Identification of L-Dopa[^{35}S] generated during metabolic labeling or enzymatic assay.** Panels (A) and (B) correspond to labeling medium samples of [^{35}S]sulfate-labeled HepG2 human hepatoma cells upon the two-dimensional thin-layer separation in the presence of synthetic L-Dopa 3-*O*-sulfate or L-Dopa 4-*O*-sulfate. Panels (C) and (D) correspond to the M-form phenol sulfotransferase-catalyzed sulfation reaction mixture samples following the separation in the presence of L-Dopa 3-*O*-sulfate or L-Dopa 4-*O*-sulfate. The protein concentration of the M-form phenol sulfotransferase used in the enzymatic assay was 0.07 mg/ml. The figure shows the autoradiographs taken from the TLC plates used for the analyses. The dashed line circles indicate the positions of carrier L-Dopa 3-*O*-sulfate or L-Dopa 4-*O*-sulfate detected by ninhydrin staining.

Identification of L-Dopa[^{35}S] Generated during Metabolic Labeling or Enzymatic Assay—Following the separation and identification of L-Dopa 3-*O*-sulfate and L-Dopa 4-*O*-sulfate described above, these two compounds were used as carriers in the identification of the L-Dopa[^{35}S] generated during metabolic labeling of HepG2 human hepatoma cells or enzymatic assay using recombinant human M-form phenol sulfotransferase. As shown in Fig. 5, upon the two-dimensional thin-layer separation (*15*), the L-Dopa[^{35}S] generated during either the metabolic labeling (panels A and B) or the enzymatic assay (panels C and D) co-migrated with L-Dopa 3-*O*-sulfate, but not L-Dopa

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4-*O*-sulfate. These results therefore demonstrated unequivocally the substrate specificity of the M-form phenol sulfotransferase being for the meta-, but not para-, hydroxyl group of L-Dopa.

It should be pointed out that, although our previous studies have shown that the sulfation of L-p-tyrosine, which lacks a meta-hydroxyl group can still occur at a low level (*5, 6*), the sulfotransferase activities toward L-Dopa in HepG2 cells or rat liver homogenates have been shown to be one or two orders of magnitude higher (*7, 9*). In view of the hypothetical role of the Dopa/tyrosine-sulfating sulfotransferase enzymes in removing excess Dopa and tyrosine by converting them to the more water-soluble sulfated forms (*4, 5*), the lower activity toward L-p-tyrosine, which is essential for protein synthesis, could have been evolved to prevent its unnecessary loss from the body. In fact, both the recombinant human M-form phenol sulfotransferase and rat liver Dopa/tyrosine sulfotransferase were shown to be nearly two orders of magnitude more active toward DL-m-tyrosine than L-p-tyrosine (*7, 9*). It has been reported that L-m-tyrosine, which can be converted to L-Dopa *in vivo* (*11, 17*), accounts for approximately 2.8% of total tyrosine circulating in blood (*18*). Therefore, the specificity of the Dopa/tyrosine-sulfating sulfotransferase enzymes, including the M-form phenol sulfotransferase, for the meta-hydroxyl group may imply their functional role in the homeostatic regulation of L-Dopa and L-m-tyrosine, which are more directly involved in the catecholamine biosynthesis (*19*), instead of L-p-tyrosine, which plays a more general role in protein synthesis.

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