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A MODIFIED ECTEOLA CELLULOSE ASSAY FOR M AND P PHENOL SULFOTRANSFERASE

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Abstract—The development and applications of a modified Ecteola cellulose, ion exchange assay for phenol sulfotransferase (EC 2.8.2.1, PST) are described. Mixtures containing dopamine or phenol and ^{35}S-labeled 3′-phosphoadenosine-5′-phosphosulfate (PAPS) were incubated with 100,000 *g* supernatant solution from human frontal cortex and applied to 0.5 × 2 cm columns of Ecteola cellulose. Dopamine sulfate was eluted with 3 ml of distilled water, while phenyl sulfate, inorganic sulfate and unreacted PAPS were eluted with successive step gradients of 5, 20 and 200 mM NH_4HCO_3. The solution volume for phenyl sulfate was 11 ml, while those for inorganic sulfate and PAPS were both 6 ml. The new assay method yielded apparent K_m values for dopamine, 3-methoxytyramine, tyramine and norepinephrine similar to those obtained by other methods. Comparison of the activities of various amine substrates at a concentration of 20 μM showed that dopamine was the preferred substrate, followed in decreasing order of relative activity by 3-methoxytyramine, norepinephrine, tyramine and octopamine. When mixed substrate inhibition of dopamine sulfation by phenol was examined, phenol was found to effectively inhibit dopamine sulfation over a range of 0.1 to 10 mM. The procedure described in this paper offers a number of significant advantages over currently available assays: these include a rapid, simple product isolation procedure and a complete, discrete separation of the radiolabeled products and reactants. This property allows the detailed study of the flux of radiolabel through the enzymatic system and also makes alternative substrate inhibition studies possible.

Phenol sulfotransferase (PST,† EC 2.8.2.1) is a soluble enzyme which catalyzes the transfer of the sulfonate (SO_3^-) moiety of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to a variety of phenolic acceptor molecules, including the biogenic amines and their metabolites [1–4]. Numerous techniques are available by which PST activity can be assessed, but all possess inherent limitations which restrict their applicability in the routine determination of sulfoconjugation, particularly of biogenic amines [1, 2, 5–8]. These problems include cumbersome product isolation procedures, or restrictive assay conditions which permit the measurement of only one form of PST, or the sulfation of one specific substrate.

Of the methods available for measuring PST activity, one of the most widely used is the radiochemical assay of Foldes and Meek [1], which utilizes differences in the solubility of the barium salts of PAPS and various phenolic acceptor molecules to determine activity. However, there are a number of shortcomings which limit the usefulness of this procedure. For example, blank values vary substantially under standard assay conditions (sometimes over a 10-fold range), varying directly with the amount of enzyme activity present in a given tissue sample. Furthermore, blank values are sensitive to changes in the ionic strength of various buffers, particularly triethanolamine and potassium phosphate. Additionally, poor recovery has been reported for various sulfated products using this technique [1, 8], making it difficult to accurately quantitate the extent to which a given molecule may be conjugated.

Borchardt *et al.* [8] have reported recently on the development of a chromatographic assay using the anion exchange medium Ecteola cellulose, which successfully remedies a number of the problems encountered using the method of Foldes and Meek [1]. However, this chromatographic procedure has its own limitations, most notably the length of time and the extremely large elution volumes required for elution of sulfated products from the Ecteola cellulose columns.

The difficulties associated with the use of the techniques described above have prompted the development of a modified version of the Ecteola cellulose assay of Borchardt *et al.* [8] which is both more rapid and is capable of quantitatively separating all reaction products from one another.

MATERIALS AND METHODS

Isolation of PST from human brain. Sections of frontal cortex from human brain were obtained at autopsy within 12 hr of death. Tissue was stored at −80° until use. Postmorten changes in enzyme activity were expected to be minimal, as it has been demonstrated previously that no correlation exists between PST activity and storage time [9]. Approximately 10–20 g of cortical tissue was thawed and homogenized with a Teflon–glass homogenizer in

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† Abbreviations: PST, phenol sulfotransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; SO_4, inorganic sulfate; DA-SO_4, dopamine sulfate; and PAP, 3′-phosphoadenosine-5′-phosphate.

5 vol. of 10 mM triethanolamine, pH 7.4, containing 0.25 M sucrose, 5 mM 2-mercaptoethanol and 500 μM $MgCl_2$. The homogenate was centrifuged at 12,000 *g* for 30 min, and the resulting supernatant fraction was recentrifuged at 100,000 *g* for 60 min. The microsomal pellet was discarded, and aliquots of the high speed supernatant solution were stored at −80°. All procedures during isolation of the enzyme were carried out at 4°. Protein was determined by the method of Lowry *et al.* [10], using bovine serum albumin as a standard.

Preparation of Ecteola cellulose. Ecteola cellulose was prepared using a modification of the procedure of Balasubramanian *et al.* [11]. In brief, the ion exchange medium was initially suspended in 20 vol./g dry weight of 0.1 N HCl for at least 30 min with frequent agitation. The cellulose was subsequently washed with deionized water and resuspended in the same volume of 0.1 N NaOH for 30 min, this latter step being repeated once. The cellulose was then decanted into a column and converted first to the formate counter-ion form by equilibration with 0.5 N formic acid and subsequently equilibrated with 5 mM NH_4HCO_3. The cellulose prepared in this manner was stored at 4° in a tightly-sealed container until use.

Assay of PST activity. Incubation mixtures routinely consisted of 10 μM dopamine (DA) or phenol in the presence of 1 μM PAP[^{35}S] (0.8 μCi/μmole), 1 mM pargyline (a monoamine oxidase inhibitor) and enzyme in a final volume of 0.5 ml. When the method of Foldes and Meek [1] was used, the reaction was terminated after a 30-min incubation by the addition of 0.1 ml of 0.1 M barium acetate, followed by the addition of 0.1 ml of 0.1 M $Ba(OH)_2$ and $ZnSO_4$. The supernatant solutions obtained after centrifugation containing ^{35}S-labeled product were retained for liquid scintillation counting. When the Ecteola cellulose procedure was employed, reactions were terminated by placing the samples in boiling water for 30 sec. Following this step, the reaction mixtures were applied to 0.5 × 2 cm columns of Ecteola cellulose which had been prepared in 5 3/4 inch pasteur pipets. Radioactively-labeled sulfated product, inorganic sulfate and PAPS were eluted from the column as described in Results.

Identification of radioactive fractions eluted from ecteola cellulose columns. The identity of the radioactive PAPS and inorganic sulfate (SO_4) eluting from the columns was determined by comparison with the elution profiles of the authentic compounds. Positive identification of the dopamine sulfate peak was established using high pressure liquid chromatography. Retention times for ^{35}S-labeled material formed in the assays were compared with that of authentic [^{3}H]dopamine sulfate synthesized by the method of Jenner and Rose [12], using a system consisting of a Waters C-18 column, an Altex 110A pump and a Bioanalytical Systems LC-4 electrochemical detector. The mobile phase consisted of 0.1 M potassium phosphate, pH 3.0, 10% (v/v) methanol, 10^{-4} M EDTA and 3×10^{-4} M sodium octyl sulfate, at a flow rate of 1 ml/min. In this system, authentic [^{3}H]dopamine sulfate possessed a retention time of 4.50 min.

Materials. 3′-Phosphoadenosine-5′-phospho[^{35}S] sulfate (0.1 to 5.0 Ci/mmole) and Formula 963 liquid scintillation mixture were purchased from the New England Nuclear Corp., Boston, MA. Triethanolamine free base, phenol and 2-mercaptoethanol were obtained from the J. T. Baker Chemical Co., Phillipsburg, NJ, while 3-methoxytyramine-HCl was obtained from the Aldrich Chemical Co., Milwaukee, WI. Magnesium chloride, triethanolamine, dopamine, tyramine, octopamine, norepinephrine and pargyline were purchased as hydrochloride salts from the Sigma Chemical Co., St. Louis, MO.

RESULTS

The elution profiles from reactions carried out in the presence and absence of 10 μM dopamine are shown in Fig. 1, A and B, respectively. As illustrated in Fig. 1A, dopamine sulfate was completely eluted

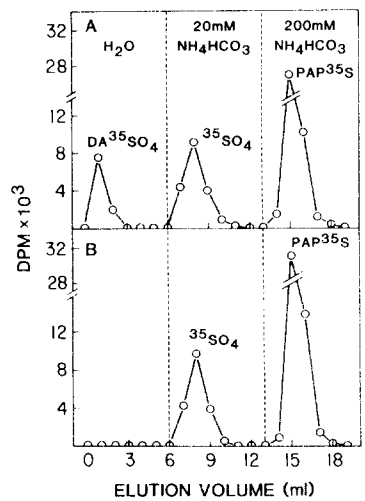

Fig. 1. Elution pattern of dopamine sulfate, inorganic sulfate and PAPS from Ecteola cellulose. Assay mixtures consisting of 10^{-3} M pargyline, 1 μM PAPS and 100,000 *g* supernatant solution were incubated in the presence (A) and absence (B) of 10 μM dopamine for 30 min at 37°. The total reaction volume, 0.5 ml, was placed over a 0.5 × 2 cm column of Ecteola cellulose, and the radioactive fractions were eluted as indicated.

from the Ecteola cellulose in the first 3 ml of the water wash, whereas SO_4 and unreacted PAPS were eluted from the column with 6 ml each of 20 mM and 200 mM NH_4HCO_3 respectively. As shown in Fig. 1B, for the blank reaction performed in the absence of the substrate dopamine, the amount of radiolabel present in the initial water wash was only slightly above background (60–120 dpm/1-ml fraction), accounting for only 0.1 to 0.3% of the total ^{35}S initially present.

Figure 2 (A and B) illustrates the elution patterns of radioactivity for reaction mixtures incubated in the presence and absence of 10 μM phenol respectively. In contrast to the sulfate conjugate of dopamine, phenyl sulfate was not removed from the column with water but required elution with 11 ml of 5 mM NH_4HCO_3. [^{35}S]Phenyl sulfate appeared in

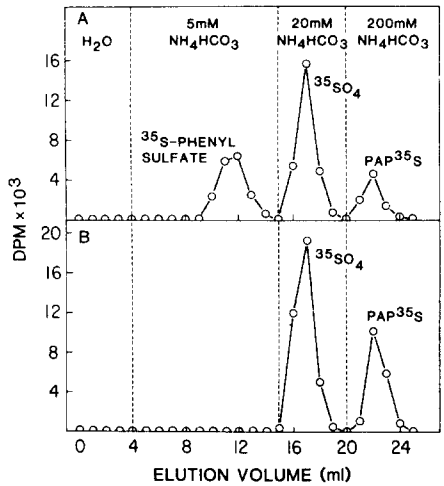

Fig. 2. Elution pattern of phenyl sulfate, inorganic sulfate and PAPS from Ecteola cellulose. All samples were prepared and eluted as in Fig. 1A and 1B, with the exception of the substitution of 10 μM phenol for dopamine in the reaction mixtures.

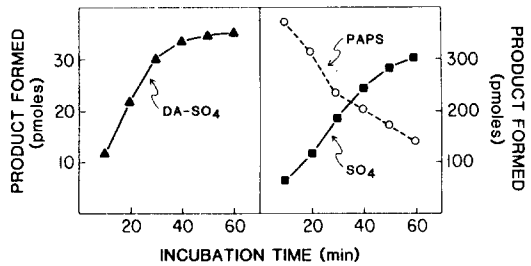

Fig. 3. Effect of incubation time on (A) the formation of dopamine sulfate (▲—▲), and (B) PAPS utilization (○—○) and inorganic sulfate production (■—■) in 100,000 g supernatant solution from human frontal cortex. Assay conditions and elutions were carried out as described in Fig. 1, A and B.

the final 5 ml of this wash. As illustrated in Fig. 2B, when phenol was omitted from the reaction mixture, only 100–420 dpm fraction was detected in the 5 mM NH_4HCO_3 elution, accounting for approximately 0.2 to 0.7% of the total PAPS present in the starting assay mixture. Recovery of radiolabel in these experiments, and for those performed with dopamine, as described above, was essentially quantitative, ranging from 96 to 99%. Although not shown, the sulfate ester of the deaminated products of dopamine, homovanillic acid, eluted from the Ecteola column in the same NH_4HCO_3 fraction as that of phenol sulfate whereas the sulfate ester of dihydroxyphenylacetic acid eluted from the column in 10–20 ml of 5 mM NH_4HCO_3 solution.

Incubations performed in the absence of 100,000 g supernatant solution resulted in approximately 3% of the total radiolabel present in the reaction mixture appearing as inorganic sulfate while the remainder was present as PAPS. No radioactivity appeared in any other fraction.

The effect of incubation time was also examined using the new assay procedure. As illustrated in Fig. 3A, DA-SO_4 formation was linear for approximately 30 min under standard assay conditions, plateauing rapidly thereafter. Inorganic sulfate production in this experiment (Fig. 3B) was linear for approximately 40 min, and accounted for almost 45% of the PAPS utilized. At the end of 60 min, approximately 70% of the PAPS was degraded. This experiment demonstrates that, in human frontal cortex preparations, inorganic sulfate accounted for the majority of PAPS utilized, while dopamine sulfate accounted for only a small percentage of the total PAPS consumed over the course of the incubation.

Figure 4 shows the relationship between protein content and reaction velocity using the Ecteola cellulose assay, with 100,000 g supernatant solution from human frontal cortex as the enzyme source. Product formation was found to vary linearly with protein content over a range of 0.2 to 1.0 mg/0.5 ml assay volume. Blank values did not vary with protein concentration.

To determine the applicability of the new assay for use with other amine substrates, the relative activities of several related substrates were compared (Table 1). At a concentration of 20 μM, dopamine was found to be the most active substrate, and was followed in decreasing order of specific activity by 3-methoxytyramine, norepinephrine, tyramine and octopamine. In addition, to verify the accuracy of the Ecteola cellulose procedure, apparent K_m values for dopamine and 3-methoxytyramine were compared using the method of Foldes and Meek [1] and the new assay procedure. As shown in Table 2, both methods yielded similar apparent K_m and V_{max} values for each substrate. In a similar fashion, the apparent K_m values for norepinephrine and tyramine determined using the new assay procedure were comparable to previously published values [4, 13].

Since it was possible to chromatographically differentiate dopamine sulfate from phenyl sulfate on the basis of their net charges using the new assay system (see Figs. 1 and 2), experiments were undertaken to evaluate the practicality of mixed-substrate inhibition studies utilizing dopamine and phenol. As illustrated in Fig. 5, phenol inhibited DA sulfation (1 μM DA) over a range of 10^{-4} M to 10^{-2} M, inhibition being essentially complete at 10^{-2} M. Since the apparent inhibition of dopamine sulfation by

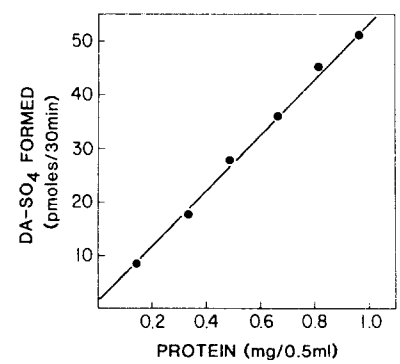

Fig. 4. Effect of protein concentration on the formation of dopamine sulfate in 100,000 g supernatant solution from human frontal cortex. Assay conditions were as described in Fig. 1, A and B.

Table 1. Human brain phenol sulfotransferase: Sulfation of various biogenic amines*

Compound	Specific activity (pmoles/30 min/mg protein)
Dopamine	58.6
3-Methoxytyramine	36.2
Norepinephrine	26.6
Tyramine	11.9
Octopamine	1.8

* PST activity was determined using the Ecteola cellulose assay as described in the text. All amine substrates were assayed at 20 μM in the presence of 1 mM pargyline and 1 μM PAPS. Incubation was initiated by the addition of 100,000 g supernatant solution from human brain and carried out at 37° for 30 min.

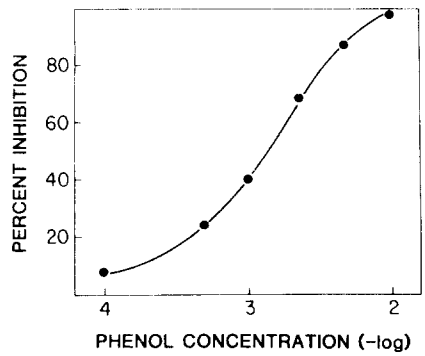

Fig. 5. Inhibition of dopamine sulfation by phenol. Assay conditions were as noted for Fig. 1, A and B, with the exception that the dopamine concentration was reduced to 1 μM. Dopamine sulfate and phenyl sulfate were eluted from the columns as described in Figs. 1 and 2 respectively. In the absence of phenol, 9 pmoles of dopamine sulfate was formed.

phenol could be caused by lack of available PAPS rather than true inhibition, the formation of DA sulfate, phenyl sulfate and inorganic sulfate, as well as overall utilization of PAPS, was determined over the range of phenol concentrations previously described (Fig. 6). In these experiments, dopamine sulfate accounted for only 1.8% of the PAPS utilized when incubations were performed in the absence of phenol. Phenyl sulfate varied from 14 to 8% of the total PAPS utilized as the phenol concentration was increased in the assay. The majority of the PAPS utilized was accounted for by formation of inorganic sulfate, which ranged from 40 to 35% of the total [^{35}S]PAPS present in the reaction mixture. Overall utilization of PAPS was similar in the blank and at the highest phenol concentrations employed. Both the production of phenyl sulfate and inorganic sulfate declined as the amount of phenol present in the incubation mixture increased.

DISCUSSION

As stated in the introduction, currently available assays for PST possess inherent drawbacks which either limit or prohibit their use in the study of biogenic amine sulfation. For example, the procedure of Segal and Mologne [14] actually measures the reverse reaction catalyzed by PST and can only use substrates with high sulfate-donating capacity, such as p-nitrophenylsulfate. The methods of Banerjee and Roy [5], as well as those of van Kempen and Jansen [6] and Wong [7] have found wide use in the general study of sulfoconjugation, but cannot be used to measure the conjugation of biogenic amines. A radiochemical method recently developed by Sekura *et al.* [15] is applicable to a wide variety of substrates, including biogenic amines, but is limited in its usefullness because time-consuming thin-layer chromatography is required to isolate reaction products prior to liquid scintillation counting.

Recent studies by Baranczyk-Kuzma *et al.* [16] and Borchardt *et al.* [8] have utilized the anion-exchanger Ecteola cellulose to assay for the sulfated products formed by PST. However, both of these procedures are extremely time-consuming in that large volumes of eluant are required to isolate the sulfated metabolites formed in the reaction. Baranczyk-Kuzma *et al.* [16] report an elution volume of 75 ml for conjugated p-nitrophenol using a four-step gradient consisting of 20, 50, 100 and 200 mM NH_4HCO_3. Determination of PAPS required elution with an additional 20 ml of 350 mM NH_4HCO_3. Using a similar method, Borchardt and co-workers [8] have reported on the isolation of the sulfate esters of several catecholamine deaminated metabolites. For example, elution of homovanillic alcohol sulfate requires a two-step gradient of 20 and 50 mM NH_4HCO_3, while homovanillic acid sulfate requires an additional gradient of 100 mM NH_4HCO_3. Under these conditions, the respective elution volumes reported were approximately 30 and 55 ml. The elution profiles of

Table 2. Kinetic constants for dopamine and 3-methoxytyramine as determined by Ecteola cellulose and Foldes and Meek assays*

Substrate	Ecteola cellulose $K_{m,app}$	Ecteola cellulose V_{max}	Foldes and Meek $K_{m,app}$	Foldes and Meek V_{max}
Dopamine	5.8	28	4.8	25
3-Methoxytyramine	1.3	54	0.9	45
Norepinephrine	12		13†	
Tyramine	108		91‡	

* PST activity was assayed as described in the text. All K_m values listed are μM; V_{max} values are expressed as pmoles/30 min.
† As reported by Roth *et al.* [13].
‡ As reported by Rein *et al.* [4].

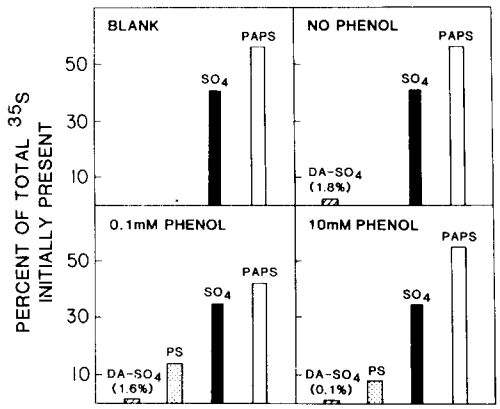

Fig. 6. Distribution of radiolabel following inhibition of dopamine sulfation by phenol. Dopamine sulfate (DA-SO_4), phenyl sulfate (PS), inorganic sulfate (SO_4) and unreacted PAPS were isolated as described in the text and assay conditions were identical to those described in Fig. 5. The blank consisted of assays performed in the absence of both acceptor molecules. Phenol concentrations employed are indicated in the upper left corner of each panel.

the sulfated catecholamines were not examined by Borchardt and co-workers [8].

In addition, the use of Ecteola cellulose to determine PST activity also offers significant improvements over the performance of the widely used method of Foldes and Meek [1]. Recovery of radiolabel is quantitative in the new procedure, which circumvents the difficulties associated with determination of the solubility and recovery of the barium salts of sulfated reaction products. Furthermore, blank values in the new assay are lower than that for the Foldes and Meek assay and often approach background levels. In addition, the barium precipitation procedure of Foldes and Meek [1] cannot differentiate between two simultaneously sulfated substrates, whereas the new assay is highly selective and capable of isolating products with different net charges. Using appropriate substrates at proper concentrations, this property allows the simultaneous measurement of two forms of PST in one sample, as well as facilitating alternative substrate inhibition analysis of the enzyme.

Under the conditions employed in this study, PST activity in 100,000 *g* supernatant solutions of human frontal cortex was linear for approximately 30 min, yet less than 9% of the PAPS initially present was utilized to sulfate dopamine. Weinshilboum and co-workers [17, 18] have suggested that nonlinearity with protein may be due to the presence of endogenous inhibitors of PST in various human tissues. To address whether endogenous inhibitors accounted for the nonlinearity we observed with time, inorganic sulfate formation and PAPS utilization were also measured using the new assay. As illustrated in Fig. 3, the high rate of PAPS utilization observed suggests that deviations from linearity in dopamine sulfate production could be accounted for by a decrease in available PAPS and or a progressive accumulation of the inhibitory reaction product 3′-phosphoadenosine-5′-phosphate (PAP). Accordingly, these results imply that tissue differences in the total rate of PAPS utilization may account for the nonlinearity of the reaction, rather than the presence of endogenous inhibitors, as previously suggested. Possible pathways for the degradation of PAPS include the enzymes 3′-nucleotidase [19], 5′-nucleotidase [20, 21], or phosphatase [22].

The procedure described in this report offers a number of significant improvements over the performance of other PST assays. The extremely low blank values encountered confer the advantage of significantly extending the sensitivity of the assay without the need for the increased use of costly [^{35}S] PAPS. Furthermore, the speed of sample preparations makes the processing of large numbers of samples feasible and the high degree of chromatographic resolution allows the determination of both product formation and substrate utilization. This latter property permits the study of the flux of radioactivity through the enzymatic system, making it possible to establish whether or not observed levels of PST activity are the result of the amount of enzyme present or the presence of competing pathways of PAPS utilization. Additionally, the selectivity of the assay may also be employed in the kinetic analysis of PST, particularly in studies of alternative substrate inhibition.

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