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RADIOISOTOPIC ASSAY FOR RAT LIVER SULFOTRANSFERASE ACTIVITY*

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Abstract—A hepatic sulfotransferase enzyme catalyzing the transfer of sulfate-(^{35}S) from 3′-phosphoadenosine-5′-phosphosulfate-(^{35}S) to *p*-nitrophenol was partially purified by differential centrifugation and $(NH_4)_2SO_4$ fractionation. Employing this enzyme, a radioisotopic procedure for assaying rat liver sulfotransferase activity is described. The enzyme activity was linear for the first 10 min of incubation and with a protein concentration up to 0·2 mg/ml of incubation mixture. Enzyme activity was present within a pH range of 5·4 to 8·4; however, maximum sulfate transfer activity occurred around pH 6·4. Seventy-one per cent of the initial enzyme activity was present after almost 2 months of storage at −75°, while the activity of enzyme stored at 4 and −20° had decreased to 32 and 30 per cent of the original activity respectively. In addition, the Michaelis constant (K_m) and maximal velocity were determined for *p*-nitrophenol, salicylamide and dehydroepiandrosterone.

The conjugation of substrates with sulfate by the liver involves several reactions which take place in the soluble fraction of cells. The sulfate must be activated by a series of reactions involving ATP which is first converted to adenosine-5′-phosphosulfate (APS) and then to 3′-phosphoadenosine-5′-phosphosulfate (PAPS). PAPS serves as the active form which transfers the sulfate to the substrate. This transfer of the sulfate from PAPS to the substrate involves several sulfotransferase (or sulfokinase) (EC 2.8.2.1) enzymes which are specific for different substrates.

A number of reports have been published describing the separation of sulfotransferases from mammalian liver [1–4], but none of these has led to a simple, rapid method for studying the sulfation of drugs via the liver *in vitro*. Recently, with the commercial availability of PAPS-(^{35}S), radioisotopic methods for assaying sulfotransferase activity of rat brain have been published by Foldes and Meek [5] and Hidaka and Austin [6]. This communication extends these studies to include a convenient radioisotopic method for assaying sulfotransferase activity in rat liver. *Para*-nitrophenol (p-NP) was chosen as a model compound because it has been well documented that p-NP is sulfated by liver sulfotransferases [1, 3, 4].

In addition, a partial purification of the hepatic sulfotransferase enzymes has been obtained to allow assessment of hepatic sulfotransferase activity of several substrates *in vitro*. The effect of storage and pH changes on the p-NP sulfotransferase enzyme and the determination of Michaelis constants (K_m) and velocities of various drug substrates are reported.

METHODS

Male Sprague–Dawley rats (170–400 g), obtained from Hiram Davies Co. (Stockbridge, Ga.), were used as a source of the liver sulfotransferase enzyme. The animals were allowed free access to food and water at all times.

3′-Phosphoadenosine-5′-phosphosulfate, tetrasodium salt-(^{35}S) (4·0–7·0 Ci/m-mole; carrier free) was obtained from New England Nuclear (Boston, Mass.) with a reported radiochemical purity of >99·5 per cent. All other reagents were the best available commercial grades. Protein concentration was determined by the method of Lowry *et al.* [7], using crystalline bovine serum albumin as the protein standard.

Preparation of sulfotransferase enzyme. Two to four rats were sacrificed by a blow on the head. The levers were excised, chilled on ice and homogenized in 2 vol. of 0·15 M KCl solution containing 0·1 M Tris–HCl (pH 7·4) employing a glass Potter-type homogenizer with a motor driven plastic pestle. All subsequent manipulations were carried out at 0–4°. The homogenates obtained from the livers were pooled and centrifuged at 9000 *g* for 20 min. Microsomes were sedimented from the 9000 *g* supernatant by centrifugation at 105,000 *g* for 95 min. The resulting supernatant was fractionated by the addition of an equal volume of a saturated solution of ammonium sulfate (Baker Chemical Co.). The enzyme was exposed to the $(NH_4)_2SO_4$ solution for approximately 15 min (without pH adjustment) before centrifugation and dialysis. Preliminary experiments revealed that maximum enzymatic activity was obtained upon fractionation of the 105,000 *g* supernatant with an equal volume of a saturated solution of $(NH_4)_2SO_4$ (50 per cent saturation) and supernatant. Thus, all subsequent experiments reported here were performed employing the 50 per cent $(NH_4)_2SO_4$ enzyme fraction.

The resultant precipitate obtained after $(NH_4)_2SO_4$ fractionation was redissolved in distilled water and dialyzed against 100 vol. of Tris–KCl buffer (pH 7·4) for 65–70 hr. The Tris–KCl buffer

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was changed once after 24 hr. The final dialysate was centrifuged at 2100 *g* for 20 min and the supernatant obtained was used as the source of the sulfotransferase enzyme. Once the enzyme was isolated and partially purified, it was stored at −75°. Aliquots of the enzyme were stored at 4 and −20° so that the effect of storage on the stability of the enzyme could be studied.

Enzyme reactions. Enzyme incubations were conducted aerobically at 37° in a Dubnoff metabolic shaking apparatus for 10 min. An initial 3-min period was allowed for the incubation mixture to equilibrate to 37° before the enzymatic reaction was begun by the addition of substrate. All substrates, with the exception of dehydroepiandrosterone, were dissolved in distilled water and added to the incubation reaction (0·5 ml) after the 3 min of equilibration time. Dehydroepinandrosterone was dissolved in propylene glycol and added as such (0·5 ml) to the incubation mixture; PAPS-(^{35}S) (0·6–1 mCi/ml, carrier free) was diluted to contain 60 μCi/ml, and 0·01 ml of the diluted solution was added to the incubation mixture.

The reaction mixture consisted of enzyme protein (0·2 mg/ml), substrate*, 3′-phosphoadenosine-5′-phosphosulfate, tetrasodium salt-(^{35}S) [PAPS-(^{35}S)], and sufficient Tris–KCl buffer (50 mM) to make a final volume of 3·01 ml. Blank incubation reactions were assayed without the addition of substrate to the incubation vials and experimental values (minus blank values) were not considered to be significant unless they were at least 50 per cent higher than concurrently run blanks. Values for the blanks averaged from 4 to 6 per cent and were never greater than 10 per cent of the total radioactivity added to the incubation vials. The variability between blanks in a typical experiment was not greater than 20 per cent.

To terminate the enzymatic reaction, 1·0 ml of 0·5 M $Ba(OH)_2$ was added to the incubation vial to precipitate the unreacted PAPS-(^{35}S). Then 1·0 ml of 0·5 M $ZnSO_4$ was added to remove the excess barium as $BaSO_4$. The reaction mixtures were transferred into centrifuge tubes and centrifuged at 500 *g* for 10 min. The $Ba(OH)_2$–$ZnSO_4$ precipitation step was repeated once more, followed by a second centrifugation at 500 *g* for 10 min. Aliquots (0·5–1 ml) of the resultant supernatant were counted by liquid scintillation counting. The scintillation fluid employed consisted of Dilufluor (Mallinckrodt Chemical Works) containing Bio-Solv (BBS-3; Beckman Instruments, Inc.) in a ratio of 5:1. Instrument counting efficiency for sulfur-35 was 92 per cent.

* p-NP and salicylamide, 0·25–1·0 × 10^{-5} moles/l.; dehydroepinandrosterone, 0·25–2·0 × 10^{-4} moles/l.

Thin-layer chromatography. Proof that p-NP sulfate-(^{35}S) was the end product in the incubation reactions in which p-NP was employed as the substrate was demonstrated by thin-layer chromatography. Incubation reactions were conducted as described above. After 10 min, the reaction was terminated either by the $Ba(OH)_2$–$ZnSO_4$ precipitation step described above, or the reaction vial was frozen immediately in a 1-propanol–dry ice bath (being subsequently thawed and stored on ice). Aliquots of the supernatants of both reaction mixtures were spotted on Silica gel plates with fluorescent indicator (No. 6060, Eastman Kodak Co.) and developed with methanol as the solvent. In this solvent system, fresh unreacted PAPS-(^{35}S) remained at the origin, as compared with ^{35}S from both reaction mixtures which appeared in a single spot exhibiting an R_f of 0·78–0·82. This R_f was in agreement with that of unlabeled p-NP sulfate (Eastman Kodak Co.) visualized by fluorescence.

RESULTS

Isolation and partial purification of the sulfotransferase enzyme. Partial purification of rat liver p-NP sulfotransferase enzyme is shown in Table 1. The specific activity of the enzyme was increased almost 2-fold by removing the 9000 *g* fraction from the homogenate (9·8 dis × 10^3 min^{-1}/mg of protein/min) and centrifuging the resulting homogenate to yield the 105,000 *g* fraction (17·7 dis × 10^3 min^{-1}/mg of protein/min). Precipitation of the 105,000 *g* fraction with a saturated solution of $(NH_4)_2SO_4$ (50 per cent saturation) produced a 16-fold increase in specific activity (162 dis × 10^3 min^{-1}/mg of protein/min) as compared to the original homogenate (100 per cent), and the $(NH_4)_2SO_4$ fraction revealed a 206 per cent recovery of enzyme activity. This occurred presumably as a result of the removal of inhibitors (e.g. sulfatase) from the mixture by the purification procedure.

Effect of incubation time and protein concentration on sulfotransferase activity. The effect of incubation time and protein concentration on p-NP sulfotransferase activity is shown in Fig. 1. The enzyme activity was essentially linear during the first 10 min of incubation (Fig. 1A). Therefore, this time was chosen as the maximum time for incubation. Likewise, the enzyme activity was linear with protein concentration up to a concentration of 0·2 mg protein/ml of incubation mixture (Fig. 1B).

Effect of pH on sulfotransferase activity. Figure 2 shows the effect of varying pH on the p-NP sulfotransferase activity. Enzyme activity was obtained within a pH range of 5·0–8·5. Maximum sulfate transfer activity occurred around pH 6·4.

Table 1. Partial purification of rat liver p-NP sulfotransferase*

Purification stage	Activity (dis × 10^{-6} min^{-1}/min)	Protein (mg)	Specific activity (dis × 10^{-3} min^{-1}/mg protein/min)	% Recovery	
				Activity	Protein
Homogenate	62·6	6358	9·8	100	100
9000 *g*	39·2	3664	10·7	60	58
105,000 *g*	35·4	2003	17·7	57	32
50% $(NH_4)_2SO_4$	129·1	795	162·4	206	13

* Sulfotransferase activity was measured at pH 6·4. Each value represents the mean of duplicate incubations minus a blank.

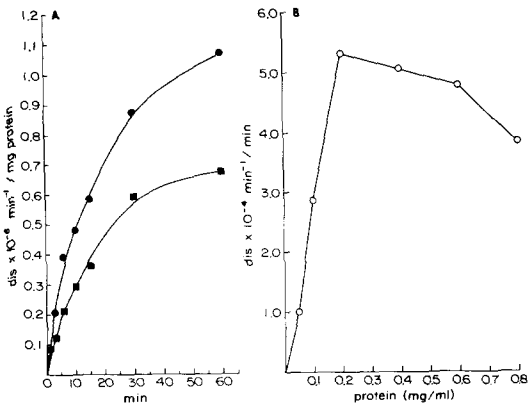

Fig. 1. (A) Effect of incubation time on p-NP sulfotransferase activity. (B) Effect of protein concentration on p-NP sulfotransferase activity. The reactions for both A and B were conducted at pH 6·4 and 37° and with a substrate (p-NP) concentration of 5 μM (■) and 10 μM (●, ○). Incubation reactions in B were conducted for 10 min. Each value represents the mean of duplicate incubations minus a blank. The enzyme fraction used was the 50 per cent $(NH_4)_2SO_4$ fraction. See text for details.

Stability of the sulfotransferase enzyme on storage. The p-NP sulfotransferase enzyme exhibited greatest stability when stored at −75° (Fig. 3). After storage of the enzyme for 24 and 53 days, the per cent of activity remaining was 88 and 71, respectively. By contrast, when the enzyme was stored at 4 or −20°, the per cent of activity remaining was approximately 66 after 24 days and 31 after 53 days.

Kinetic parameters of sulfotransferase activity. Figure 4 shows a double reciprocal plot of the incorporation of sulfate-(^{35}S) from PAPS-(^{35}S) into p-NP. Reciprocal velocities were plotted against reciprocal substrate concentrations, the points were fitted to the line by the method of least squares [8], and the kinetic parameters of the Michaelis–Menten equation were calculated according to Lineweaver and Burk [9]. As determined from the plot, the Michaelis constant (K_m) for the p-NP sulfotransferase enzyme was 0·014 mM and the velocity was 19·1 dis × 10^4 min^{-1}/mg of protein/min.

Similar Lineweaver–Burk reciprocal plots were obtained for one other drug substrate (salicylamide) and one hormonal substrate (dehydroepiandrosterone), both of which are known to undergo sulfate conjugation *in vivo* (Table 2). The K_m for salicylamide was 0·009 mM, while the K_m for dehydroepiandrosterone was 0·062 mM. The velocities for these two substrates were 5·2 and 6·6 dis × 10^4 min^{-1}/mg of protein/min respectively. However, no detectable sulfotransferase activity was obtained when morphine (sulfate, salt) was employed as the substrate.

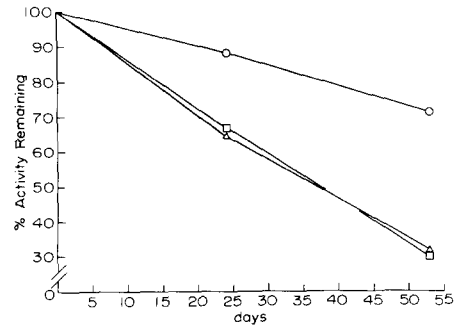

Fig. 3. Stability of the p-NP sulfotransferase enzyme on storage. The enzyme was stored at 4° (△), −20° (□) and −75° (○). Incubation reactions were conducted at pH 6·4 and 37° for 10 min.

DISCUSSION

The purpose of this communication is to report a simple, rapid radioisotopic method for studying the sulfation of drugs via the liver *in vitro*. Such a method is desirable, since the isolation and separation of sulfate metabolites formed by the liver *in vivo* and excreted in the urine are time-consuming processes. Thus, it would be beneficial to use sulfotransferase activity *in vitro* as a means of: (1) studying the sulfate conjugation of drugs by the liver, and (2) assessing the interaction of more than one drug at the metabolic level. For this work, p-NP was chosen as a model compound because it has been well documented that this compound is sulfated by liver sulfotransferases [1, 3, 4].

Although others have purified liver sulfotransferases 70 to 2000-fold [1, 4], devising an assay based on enzyme with high specific activity was not the intent of this work. On the contrary, if one desires an initial means of evaluating the sulfate conjugation of drugs *in vitro* to correlate with sulfate transfer

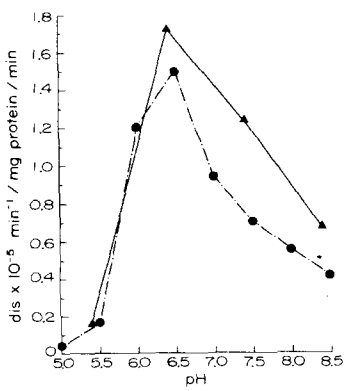

Fig. 2. Effect of pH on p-NP sulfotransferase activity. Each line represents the results obtained by using a different batch of the sulfotransferase enzyme isolated separately on two different occasions. Incubation reactions were conducted at 37° for 10 min and with a substrate concentration of 10 μM.

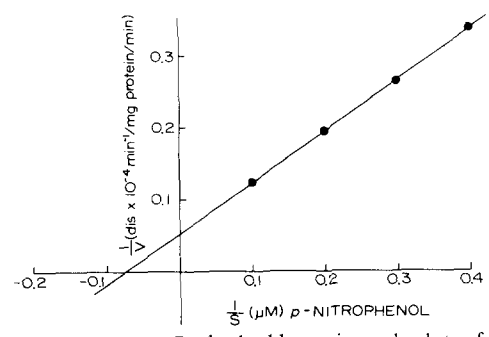

Fig. 4. Lineweaver–Burk double reciprocal plot of the incorporation of sulfate-(^{35}S) from PAPS-(^{35}S) into p-NP. The reactions were conducted at 37° for 10 min at pH 6·4 and with the substrate concentrations shown in the figure. The K_m and velocity obtained from the plot are reported in Table 2. Each point represents the mean of two to three assays.

Table 2. Substrate specificity of partially purified p-NP sulfotransferase

Substrate	K_m* (mM)	Velocity (dis × 10^{-4} min^{-1}/ mg protein/min)
p-Nitrophenol	0·014	19·1
Salicylamide	0·009	5·2
Morphine†		
Dehydroepiandrosterone	0·062	6·6

* The K_m for p-NP was determined at pH 6·4, while the K_m for salicylamide and dehydroepiandrosterone were assayed at pH 7·0.

† Morphine (sulfate salt) was not conjugated within the range of substrate concentration (1×10^{-4}–8×10^{-3}) assayed.

activity *in vivo*, then it is preferable to isolate liver sulfotransferases as a group similar to the way liver microsomal enzymes have been isolated. Once information from this type of study has been obtained, then it may be desirable to further isolate, purify and crystallize individual enzymes with high specific activities.

The p-NP sulfotransferase enzyme isolated here is stable when stored for 2 months at −75°. Bannerjee and Roy [3] found that guinea pig sulfotransferase enzymes retained their activity for many months when stored at −30° in the presence of 0·03 M EDTA, at pH 7·5. In addition to sulfotransferases, the liver contains sulfate-activating enzymes and sulfatases which may act as inhibitors of sulfotransferase activity [10–12]. In the present isolation procedure, a number of these inhibitors were apparently removed, since the recovery of enzyme activity was 206 per cent as compared to the original enzyme activity of the homogenate.

That the isolation and partial purification procedure provides a useful means of assessing sulfate conjugation activity *in vitro* was demonstrated by the observation that the crude sulfotransferase enzyme is capable of transferring sulfate-(^{35}S) not only to p-NP but to other substrates such as salicylamide and dehydroepiandrosterone which are known to undergo sulfate conjugation *in vivo*. The observation that morphine was not easily sulfated by this enzyme suggests that the conditions (pH, buffer, etc.) were not correct for this conjugation reaction. It may be necessary to further purify the enzyme to obtain an enzyme preparation with greater specificity and to remove endogenous inhibitors of the reaction.

A number of workers have purified liver sulfotransferase from different species. Nose and Lipmann [1] reported the partial separation of sulfotransferases of rabbit liver that were responsible for the formation of p-NP sulfate and estrone sulfate. The present work is in agreement with these authors, since the p-NP sulfotransferase enzyme isolated in our laboratory will sulfate dehydroepiandrosterone, and preliminary data indicate that the enzyme will also sulfate estrone.

An enzyme that catalyzes the sulfation of L-tyrosine methyl ester and tyramine was purified approximately 70-fold from female rat livers by Mattock and Jones [2]. These authors were able to demonstrate that the enzyme responsible for the sulfation of L-tyrosine methyl ester and tyramine was different from that which was responsible for the sulfation of p-NP.

Bannerjee and Roy [3] isolated a phenol sulfotransferase from guinea pig liver which was free of any detectable steroid sulfotransferase activity. They reported a pH optimum of 5·8 for p-NP sulfotransferase, while the enzyme reported here appears to yield maximum activity around pH 6·4. In addition, they were able to partially separate dehydroepiandrosterone sulfotransferase and estrone sulfotransferase activity and demonstrated that both of these enzymes were capable of sulfating p-NP and 2-naphthylamine. McEvoy and Carroll [4] have described the purification (approximately 2000-fold) from male rat liver of an enzyme that catalyzes the sulfation of simple phenols and which has no activity toward steroids, aliphatic alcohols, aromatic amines and L-tyrosine methyl ester.

In conclusion, the crude p-NP sulfotransferase enzyme has been isolated several times with reproducibility. The fact that sulfur-35 labeled PAPS allows one to measure only sulfotransferase activity and not be concerned with other enzymes (ATP-sulfurylase and APS phosphokinase), which are present when a PAPS-generating system is employed, removes variables and makes this method more specific for assaying sulfotransferase activity. This specificity combined with stability of the enzyme so that it can be stored for several months and the simple, rapid radioisotopic method for assessing hepatic sulfotransferase activity *in vitro* make the present procedure a useful means for studying the sulfation of a number of drug substrates *in vitro*.

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