Biochemical Pharmacology, Vol. 31, No. 18, pp. 2949–2954, 1982.
Printed in Great Britain.

0006-2952/82/182949-06 $03.00/0

SULFATION OF MINOXIDIL BY LIVER SULFOTRANSFERASE

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(*Received* 30 *November* 1981; *accepted* 25 *February* 1982)

Abstract—The 100,000 *g* supernatant fraction of rat liver homogenate contains a sulfotransferase activity which catalyzes the sulfation of minoxidil. Synthetic minoxidil *N-O* sulfate and the enzyme synthesized product had identical chromatographic characteristics on high pressure liquid chromatography. Minoxidil sulfate, which yields minoxidil when treated with sulfatase, was slowly hydrolyzed in water. Several *N*-oxides of other heterocycles, including several other pyrimidines, triazines and imidazoles, were also substrates for this sulfotransferase.

Minoxidil (Fig. 1), an orally effective hypotensive agent, has an extended duration of effectiveness in a number of species, including man [1, 2, a review]. The pharmacologic half-life of the drug exceeds the serum half-life [1, 3]. In addition, the onset of the hypotensive activity is delayed by 30–90 min following drug administration [1, 2]. For these reasons, the metabolism of minoxidil has been a subject of continuing interest. Reports [4, 5] and observations† have suggested that the delayed effects of minoxidil might be due to a metabolic transformation of the minoxidil molecule.

Extensive studies of the metabolism of minoxidil in rat, monkey and dog, as well as man, have been reported by Thomas and co-workers [3, 6, 7]. Metabolites of minoxidil, as determined in those studies, have been synthesized and tested for hypotensive activity. None of these compounds has demonstrated either a hypotensive activity approaching that of monoxidil or an immediate lowering of blood pressure in the various models employed.

With the continuing hint of a metabolic conversion of minoxidil to an active metabolite, we pursued further the metabolic study of monoxidil. Results of recent studies from this laboratory have noted the presence of an anionic radioactive metabolite which had been isolated from bile of rats treated with [^{14}C]monoxidil.‡ Subsequent to this, it was observed that the hypotensive activity of minoxidil was decreased markedly by the pretreatment of rats with 4-acetamidophenol,§ a possible scavenger of active sulfate groups *in vivo* [8, 9]. These results suggested that minoxidil might be the substrate for a sulfotransferase activity in which minoxidil sulfate was the product. This report describes the initial characterization of a sulfotransferase enzyme activity from rat liver which *in vitro* catalyzes the formation of the *N-O* sulfate of minoxidil and several other pyrimidine-, pyridine-, triazine-, and imidazole-*N*-oxides.

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† D. W. DuCharme and M. G. Wendling, unpublished observations.

‡ G. A. Johnson and K. J. Barsuhn, manuscript in preparation.

§ M. G. Wendling and J. E. Rogers, unpublished observations.

MATERIALS AND METHODS

Chemicals and solvents were obtained from the following commercial sources: *p*-nitrophenol (Eastman Organic Chemicals, Rochester, NY); 0.3 M $Ba(OH)_2$ solution, sulfatase (Type VI), and bis-tris propane [1,3-bis[tris(hydroxymethyl)-methylamino]propane] (Sigma Chemical Co., St. Louis, MO); 3′-phosphoadenosine-5′-phosphosulfate (PAPS), 25.1 A_{260} units/mg (PL Biochemicals, Milwaukee, WI); dimethylformamide (DMF, Burdick & Jackson, Muskegon, MI); and PAPS[^{35}S], 2.14 Ci/mmole, and Aquasol-2 (New England Nuclear Corp., Boston, MA). All compounds tested in the sulfotransferase assay were obtained from The Upjohn Co.

All chemicals and solvents were used without additional purification.

Enzyme isolation. Livers were excised from male Upjohn Sprague–Dawley rats (160–180 g), chilled on ice, and then homogenized for 1 min in 4 vol. of 0.25 M sucrose containing 10 mM KPO_4 (pH 7.3), employing a Polytron 10 homogenizer (Brinkmann Instruments, Westbury, NY). The homogenates were centrifuged at 15,000 *g* for 10 min. The resulting supernatant fraction was then centrifuged at 100,000 *g* for 60 min. This supernatant fraction was used as the enzyme source in most of the studies in this report. In one study this supernatant was further fractionated with ammonium sulfate. The residue from the 0–50% ammonium sulfate fraction was resuspended in water and dialyzed for 40 hr against three changes of 2 liters of the sucrose phosphate buffer. The final dialysate was centrifuged to remove the accumulated residue. The supernatant fraction was divided into 1-ml portions and stored at −70° in polypropylene tubes. Protein content of the enzyme preparations was determined by the method of Lowry *et al.* [10].

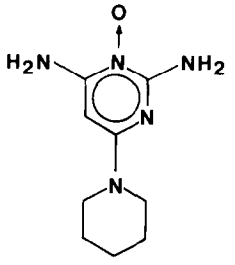


Fig. 1. Minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide).

Enzyme assay. Enzyme incubations were carried out in glass 12 × 75 mm test tubes; the final volume of incubate was 200 μl. All additions were made, with mixing, on ice. The incubation time was 30 min; all incubations were done in an oscillating water bath at 37°. The incubation mixture consisted of 62 mM bis-tris propane, 0.5 A_{260} units of PAPS (containing 1–4 × 10^5 cpm PAPS[^{35}S]), enzyme and the sulfate acceptor molecule. Compounds that were not soluble in the buffer were dissolved in dimethylformamide; a final concentration of 5% dimethylformide did not affect enzyme activity. Concentrations listed are final concentrations. All samples were run in duplicate. Blanks contained all the components except the sulfate acceptor. Reactions were stopped by freezing or by the addition of $Ba(OH)_2$ [11, 12].

Product assay. Inorganic sulfate and excess PAPS were removed by a modification of the $Ba(OH)_2$ precipitation technique [11]. One milliliter of 0.3 N $Ba(OH)_2$ was added, with mixing, to the incubation tubes, followed by 0.33 ml of 0.5 M $ZnSO_4$. After thorough mixing, the tubes were centrifuged for 20 min at 1200 *g*. A 200-μl aliquot of the clear supernatant fluid containing the radiolabeled sulfate product was removed and added to 10 ml of Aquasol-2. Radioactivity in the aliquot was measured by scintillation counting in a Packard TriCarb liquid scintillation spectrometer using the ^{14}C channel.

In separate experiments the $Ba(OH)_2$–$ZnSO_4$ step was found to precipitate > 99% of the radiolabeled PAPS.

Identification of products of incubation. Reverse phase high pressure liquid chromatography (HPLC) was employed to identify the product of the incubation of minoxidil with the 100,000 *g* liver supernatant fraction and PAPS[^{35}S]. An Altex (Altex Scientific, Inc., Berkeley, CA) model 312 gradient chromatograph was used to deliver a solvent consisting of 0.01 M NaH_2PO_4 buffer, pH 2.8/acetonitrile (85:15). An aliquot of the incubation mixture was diluted with the solvent, and an aliquot of the diluted incubation mixture was injected onto the column via a Rheodyne (Rheodyne, Inc., Berkeley, CA) 100-μl injection valve. The chromatograph was run at ambient temperature at 3 ml/min and at a pressure of 2000 psi. The substrate and product were resolved on a stainless steel Rheodyne column, 25 cm × 4.6 mm, i.d., packed with LiChrosorb RP-8, 10 μm (E. Merck, Darmstadt, West Germany). A MPLC RP-8 (Rheodyne) guard column preceded the analytical column. The u.v. absorption at 280 nm was recorded (model 385 recorder, Linear Instrument, Houston, TX). The eluate from the flow cell was collected with a fraction collector (model FC-80K, Gilson Medical Electronics, Middleton, WI). Fractions were added to 10 ml of Aquasol-2, and the radioactivity was determined by scintillation counting.

RESULTS

Initial studies demonstrated the presence of sulfotransferase (EC 2.8.2.1; 3′-phosphoadenylylsulfate:phenol sulfotransferase) activity in the 0–50% ammonium sulfate fraction from rat liver using *p*-nitrophenol (50 mM) as the sulfate acceptor. The optimal pH (6.4 to 7.0) for the conversion of *p*-nitrophenol to *p*-nitrophenol sulfate agreed with that previously reported [11, 12]. At pH 6.4, minoxidil (15 mM) was not a substrate for this enzyme. An expanded pH study over the range of 6.0 to 9.0 indicated that minoxidil was a substrate for the enzyme at pH 7.5 or higher. The pH dependence of minoxidil sulfate[^{35}S] formation is compared with that of *p*-nitrophenol sulfate[^{35}S] in Fig. 2. The pH curve for *p*-nitrophenol is distorted since, under the conditions of this assay, substrate became rate-limiting. Formation of minoxidil sulfate[^{35}S] at pH 8.0 was dependent upon protein concentration (Fig. 3). Further, the yield of product increased with increasing minoxidil concentrations (Fig. 4); minoxidil and *p*-nitrophenol concentrations above 15 and 3 mM, respectively, produced inhibition of enzyme activity. The enzyme-catalyzed formation of minoxidil sulfate[^{35}S] and of *p*-nitrophenol sulfate[^{35}S] was maximum at 60 min of incubation time. Mg^{2+} (10 mM), had no effect upon the sulfation of 15 mM minoxidil at pH 8.0. Enzyme activity in the 100,000 *g* supernatant fraction was maintained for several months during storage at −76°. In contrast, enzyme activity in the 0–50% ammonium sulfate fraction was lost during storage under the same conditions.

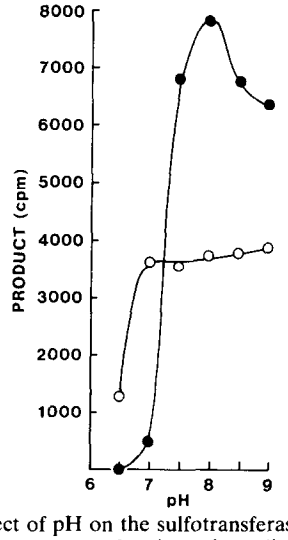


Fig. 2. Effect of pH on the sulfotransferase activity in the 100,000 *g* supernatant fraction of rat liver homogenate. Key: (○) *p*-nitrophenol, 3 mM; and (●) minoxidil, 15 mM. Each point is the average of duplicate determinations. Incubation conditions are described in Materials and Methods. Bis-tris propane (62 mM) was utilized at each point; the pH of the buffer was adjusted with HCl.

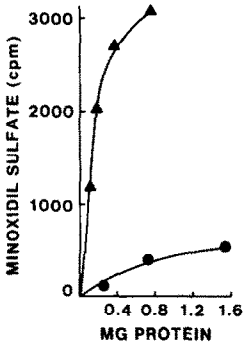


Fig. 3. Effect of increasing amounts of protein on the sulfation of minoxidil. Minoxidil (3 mM) was the substrate in each mixture. Key: (▲) 100,000 *g* supernatant fraction; and (●) 0–50% ammonium sulfate fraction of the 100,000 *g* supernatant fraction. Each point is the mean of duplicate determinations.

Although the Ba–Zn precipitation step removed > 99% of the labeled substrate, confirmation was required that the radioactivity remaining after precipitation was minoxidil sulfate[^{35}S]. Enzymatically synthesized minoxidil sulfate[^{35}S] had the same mobility on an RP-8 reverse phase column as synthetic minoxidil sulfate (Fig. 5). Confirmation of the structure of chemically synthesized minoxidil sulfate was unambiguously assigned as a result of X-ray crystallography. The identity of the enzyme-synthesized product with the chemically synthesized minoxidil sulfate was demonstrated by mass spectrographic analyses of the trimethyl silyl derivatives of the two products.* Mass spectra of the derivatives of enzymatically and chemically derived minoxidil sulfate were identical.

Incubation of the minoxidil sulfate[^{35}S] with a commercial bacterial sulfatase produced a time-dependent decrease in minoxidil sulfate[^{35}S] (Fig. 6). This effect was blocked by boiling the sulfatase prior to adding the protein to the incubation mixture.

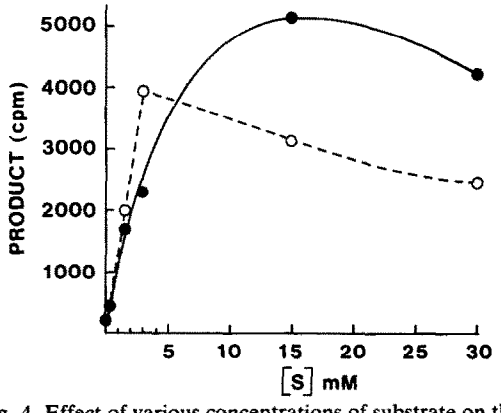


Fig. 4. Effect of various concentrations of substrate on the formation of sulfate product. Key: (○) *p*-nitrophenol at pH 7.0; and (●) minoxidil at pH 8.0. Each incubation mixture contained 0.22 mg protein from the 100,000 *g* supernatant fraction.

* J. M. McCall, manuscript in preparation.

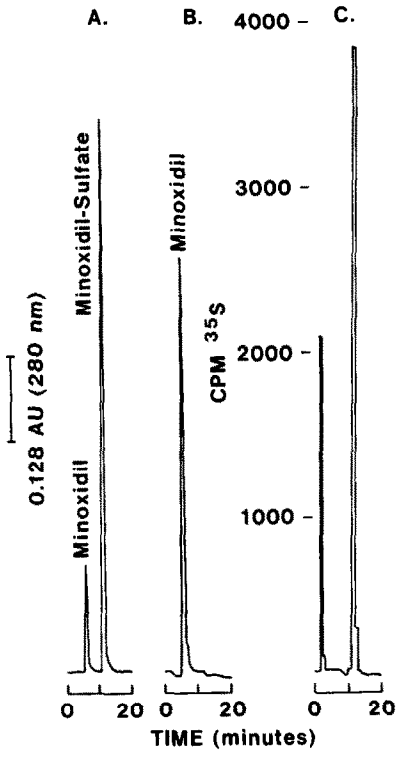


Fig. 5. Chromatographic resolution of minoxidil and minoxidil sulfate. (A) Resolution of minoxidil and minoxidil sulfate standards using the chromatographic conditions described in Materials and Methods. (B) Chromatography of an aliquot of an incubation mixture containing minoxidil, PAPS[^{35}S], enzyme and buffer as described in Materials and Methods; unlabeled PAPS was omitted. (C) Radioactive profile of fractions collected from the aliquot in B. The initial radioactive peak contains unreacted PAPS[^{35}S].

This sulfotransferase activity is not limited to minoxidil. The ability of other pyrimidine-, as well as pyridine-, triazine- and imidazole-*N*-oxides to serve as substrates was investigated using the soluble

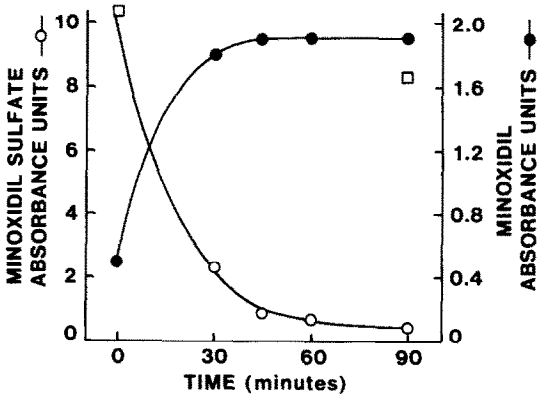
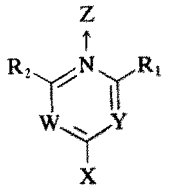
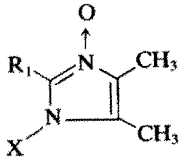


Fig. 6. Effect of sulfatase on minoxidil sulfate. Each incubation mixture contained 25 μl of 0.5 M Tris, pH 7.1, 20 μl of sulfatase and 50 μl of 0.01 M citric acid containing minoxidil sulfate, 1 mg/ml in a total volume of 200 μl. Incubation was at 37°. Peak height at 254 nm was measured following separation on HPLC. Key: (○) minoxidil sulfate; (●) minoxidil; and (□) minoxidil sulfate in incubation mixture with boiled sulfatase.

Table 1. *N*-Oxide sulfotransferase activity with heterocyclic *N*-oxides



A



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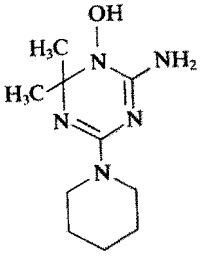

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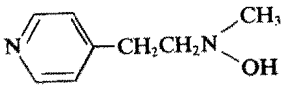

D

	Structure	Z	R_1	R_2	W	X	mM	% Converted
Pyrimidines (Y = N, W = CH)	A							
1 Minoxidil		0	$-NH_2$	$-NH_2$	—CH	Piperidino	30	7.9
2			$-NH_2$	$-NH_2$	—CH	Piperidino	30	0
3		0	Acetylamino	Acetylamino	—CH	Piperidino	10	0.4
4		0	$-NH_2$	$-NH_2$	—CH	4′-Hydroxypiperidino	15	1.4
							30	5.3
5		0	$-NH_2$	$-CH_3$	—CH	Morpholino	15	3.3
6		0	$-NH_2$	$-NH_2$	$-C-NH_2$	Piperidino	15	0
Pyridines (Y = W = CH)	A							
7		0	—H	—H	—CH	$-OCH_3$	10	2.8
8		0	$-CH_3$	—H	—CH	—Cl	5	4
9		0	—H	—H	—CH	$-NO_2$	10	0
10		0	$-CH_3$	—H	—CH	$-NO_2$	10	0
Triazines (Y = W = N)	A							
							15	1
11*		0	$-NH_2$	$-NH_2$	N	$-N-(CH_2CH=CH_2)_2$	7.5	0.8
12*		0	$-NH_2$	*p*-Chlorobenzyl—	N	$-N-(CH_2CH=CH_2)_2$	30	0.2
13*		0	$-NH_2$	$-CH_3$	N	Morpholino	30	0.8
Imidazoles	B							
							7.5	5.6
14			CH_3CH_2-			—OH	1.5	15.8
15			$-CH_3$			—OH	15	50
16			$-CH_3$			—H	30	35
Others								
17	C						30	0
18	D						15	8.4

* The pH of the incubation mixture was 7.5; all other compounds were tested as described in Materials and Methods, pH 8.0.

liver preparation and the Ba–Zn precipitation assay. The variety of structures represented in Table 1, while not extensive, provides an indication that the heterocyclic *N*-oxides are generally substrates for this enzyme activity. Presumably, all of these heterocycles are conjugated via the oxygen of the *N*-oxide. The pH maximum for the *N*-oxide sulfotransferase activity with each substrate was 8.0 to 8.5, except for the triazine substrates for which a pH 7.5 maximum was found. In addition, the only hydroxylamine-containing compound tested was also a substrate for the sulfotransferase at pH 8.0.

DISCUSSION

The characteristics of the various enzymes with sulfotransferase activity and the wide range of substrates for these sulfotransferases have been reviewed extensively [13–15]. Each of these reviews emphasizes the difficulty in characterizing the individual sulfotransferase activities which is due, in part, to the multiplicity of the substrate or sulfate acceptor molecules and to the lability of the various activities, especially upon storage [16, 17]. The characterization which has been done has been limited largely to a demonstration of the homogeneity of a specific band of protein on disc gel electrophoresis and to the elimination of enzyme activity directed toward specific sulfate acceptors.

The problem of defining specificity with the sulfotransferases was clearly demonstrated by Lyon and Jakoby [18] who purified hydroxysteroid sulfotransferase to homogeneity upon disc gel only to discover that alcohol sulfotransferase activity also resided in that protein band. More recently, Sekura and Jakoby have isolated to homogeneity from rat liver three aryl sulfotransferases [19, 20], one of which catalyzes the transfer of sulfate to simple phenols, organic hydroxylamines, catecholamines, hydroxyindoles including serotonin, and amino acids [20]. Activity of this enzyme, aryl sulfotransferase IV, is limited at pH 5.5 to the sulfation of phenols; at pH values of 7.9 and 9.0 the substrate affinities of the other acceptor molecules were also noted [20].

In the previous study, aryl sulfotransferase IV catalyzed the sulfation of the single organic hydroxylamine tested [20]. Wu and Straub [21] have demonstrated the sulfation of *N*-hydroxy-2-acetylaminofluorene, also an organic hydroxylamine, by a similarly homogeneous rat liver sulfotransferase preparation; some of the characteristics of the latter enzyme preparation conflict with those described by Sekura and Jakoby [20]. Nevertheless, the products in each case result in an *N-O* sulfate, similar to that produced upon sulfation of an *N*-oxide; the similarity in the products from the two substrates can be better understood when the *N*-oxide is viewed as a tautomer of the hydroxylamine. Activity demonstrated in this study with the *N*-oxide substrates would then coincide with that of aryl sulfotransferase IV. Several points can be raised in support of that hypothesis: (1) phenols have principal activity at a pH less than that for other substrates; (2) the hydroxylamine also serves as a substrate at or near pH 8; and (3) both the phenols and minoxidil produced substrate inhibition at higher substrate concentrations (Fig. 4). As reported for various phenols with aryl sulfotransferase IV [20], the *N*-oxides also showed a wide range of affinities for the enzyme as indicated by the yield of product (Table 1).

The assay used in this study to demonstrate the transfer of the labeled sulfate from PAPS[^{35}S] to the *N*-oxide acceptor does provide an indication of the enzyme activity. The identical chromatographic mobilities of chemically synthesized minoxidil sulfate and enzymatically synthesized [^{35}S]minoxidil sulfate, together with the mass spectral comparison of these two products support the validity of the assay methodology.

Results summarized in Fig. 4 show the substrate inhibition of the sulfotransferase activity at higher concentrations of both minoxidil and *p*-nitrophenol. Similar substrate inhibition of the sulfotransferases by phenols has been noted previously [19, 20]. Concentrations of substrates in the table were chosen primarily from solubility data; whether that concentration was on the ascending or inhibitory portion of the concentration curve was not determined (cf. compounds 5 and 14, Table 1). Consequently, the percent conversion indicated in the table may or may not be the maximum produced by the enzyme activity under the conditions of the assay.

The sulfate is conjugated to minoxidil, the substrate or acceptor molecule, on the *N*-oxide (Fig. 7). This has been determined by X-ray crystallography. The molecular conjugation of sulfate[^{35}S] to the other substrates in Table 1 was not similarly defined. However, failure of the desoxy derivative of minoxidil, i.e. reduced minoxidil [6] (compound 2, Table 1), to serve as substrate, as well as the abilities of several other *N*-oxides in Table 1, molecules in which the *N*-oxide is the only functional group (compounds 7, 8 and 16), to serve as substrates, also support the *N*-oxide as the site of the sulfate addition. The extent of the sulfation noted in the table may be only an estimate of the activity due to the possible instability of the resulting *N-O* sulfates. Marked lability of the *N-O* sulfates formed by the sulfation of the substituted hydroxylamines, including *N*-hydroxyacetylaminofluorene [9, 21, 22] and *N*-hydroxyphenacetin [23], has been noted.

A full assessment of the substrate specificity for the sulfotransferase activity cannot be made from the limited number of substrates listed in the table. Results show that sulfation of the *N*-oxides does

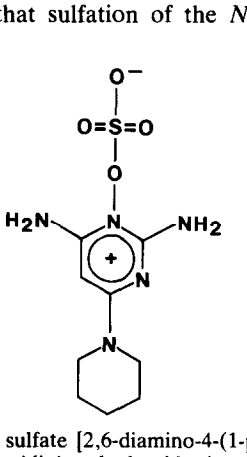

Fig. 7. Minoxidil sulfate [2,6-diamino-4-(1-piperidinyl)-1-(sulfoxy)pyridinium hydroxide, inner salt].

occur in the heterocyclic structures studied, i.e. pyrimidines, pyridines, triazines, and imidazoles. A pH maximum of 7.5 for the triazine-*N*-oxides was the only deviation from the standard assay in this group of substrates. Additional points which reflect an order of specificity in the enzymic activity were noted: the two 4-nitropyridine *N*-oxides were not substrates for the sulfotransferase; ring substituents in both the pyrimidine and the triazine groups affected the role of these compounds as substrates; and addition of an amino group at the 5-position in the pyrimidine ring of minoxidil erased substrate activity. Compound 18, a substituted hydroxylamine, was a sulfate acceptor at pH 8.0 in this system; the assay was completed in the previously cited studies with the hydroxylamines at 7.0 [20, 21].

An accurate indication of molar concentration of PAPS in the incubation mixture was not possible due to: (1) the availability of the PAPS in units rather than by weight, and (2) an approximate purity of 90%. Because of this uncertainty in the PAPS concentration, the product formed (Table 1) was quantitated as a percent conversion of the PAPS[^{35}S]. Using the purity estimate provided by the supplier, the approximate PAPS concentration in the incubation mixture was 15 μM.

Minoxidil *N-O* sulfate[^{14}C] has been isolated from the bile of rats following i.v. administration of minoxidil[^{14}C]; the *in vitro* enzyme-catalyzed sulfation of the pyrimidine *N*-oxides, including minoxidil, does then provide a measure of confirmation of its metabolic disposition *in vivo*. In contrast to minoxidil, minoxidil sulfate has a direct vasodilator activity when administered to dogs and rats;* thus, this derivative of minoxidil is a candidate for the active metabolite of minoxidil. Interestingly, 2,4-diamino-6-diallylaminotriazine-3-oxide (compound 11, Table 1), which also has potent and long-lasting vasodilator activity [24], is also a substrate for the sulfotransferase.

This demonstration of the enzyme-catalyzed sulfation of *N*-oxides extends the spectrum of sulfotransferase activities. The full role of this aryl sulfotransferase activity in the disposition of xenobiotics is yet to be investigated. However, the activity described in this report does provide an additional direction for those studies.

* D. W. DuCharme, M. R. McCandlis, J. M. McCall, F. G. Robinson, K. J. Barsuhn and G. A. Johnson, unpublished observations.

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