Hepatic Metabolism of N-Hydroxy-N-methyl-4-aminoazobenzene and Other N-Hydroxy Arylamines to Reactive Sulfuric Acid Esters

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SUMMARY

Hepatic cytosols catalyzed a 3’-phosphoadenosine 5’-phosphosulfate (PAPS)-dependent O-sulfonation of N-hydroxy-N-methyl-4-aminoazobenzene (N-HO-MAB) and several other N-hydroxy arylamines. The presumed product from N-HO-MAB, N-methyl-4-aminoazobenzene-N-sulfate, reacted with added guanosine to yield N-[(guanosin-8-yl)-N-methyl-4-aminoazobenzene, with methionine to form a sulfonium derivative that decomposed to yield 3-methylmercapto-N-methyl-4-aminoazobenzene, and with ribosomal RNA to give a bound derivative. N-Methyl-4-aminoazobenzene was converted to N-[(guanosin-8-yl)-N-methyl-4-aminoazobenzene-N-sulfate (or the nitrobenzene ion from this unstable ester) was reduced by N-HO-MAB to form N-methyl-4-aminoazobenzene; the N-HO-MAB was oxidized, probably through a nitrotronic intermediate, to yield products that included N-hydroxy-4-aminoazobenzene and formaldehyde. An analogous reaction was noted between N-benzoyloxy-N-methyl-4-aminoazobenzene and N-HO-MAB in the absence of cytosol and PAPS.

Hepatic N-HO-MAB sulfotransferase activities were in the order: male rat > female rat, male rabbit, male guinea pig, male mouse > male hamster. Male rat kidney and small intestine cytosols had low activities; the other tissues studied had little or no activity. Hepatic sulfotransferase activities for N-HO-MAB and N-hydroxy-N-acetyl-2-aminofluorene displayed different pH optima and inhibitor and activator responses.

The rates of PAPS-dependent rat liver cytosol-catalyzed esterification of N-hydroxy-N-ethyl-4-aminoazobenzene, N-hydroxy-4-aminoazobenzene, and N-hydroxy-1- and 2-naphthylamine were 20 to 50% of that for N-HO-MAB. Activities for trans-N-hydroxy-4-aminothiobenzene, N-hydroxy-4-aminoazobenzene, and N-hydroxy-N-methyl-N-benzylamine were not detected.

No microsomal reduced nicotinamide adenine dinucleotide-dependent reduction or reduced nicotinamide adenine dinucleotide phosphate-dependent oxidation or cytosolic transferase reactions for N-HO-MAB, except the above-described PAPS-dependent reaction, were detected in rat liver.

INTRODUCTION

The metabolic N-oxidation of carcinogenic amines and arylamines appears to be a necessary step in their conversion to ultimate carcinogenic derivatives (48). Two hepatic microsomal enzymes, a cytochrome P-450-dependent oxidase (21, 33, 69, 73) and a flavoprotein amine oxidase (33, 77, 78), have been implicated as catalysts for these oxidations. The N-hydroxy amines and N-hydroxy amides are then subject to further hepatic metabolism. A NADPH-dependent microsomal N-hydroxy amine reductase and a NADPH- and oxygen-dependent microsomal N-hydroxy amine oxidase catalyze the reduction and oxidation, respectively, of a number of N-hydroxy amines (32, 34, 53). A microsomal deacetylase for N-HO-AAF3 has been demonstrated in the livers of a number of species (26).

Hepatic cytosols from rats and some other species contain N-hydroxy arylacylamide-dependent N-hydroxy arylamine acetytransferase (6, 7, 36), ATP-dependent seryltransferase (66, 67), and PAPS-dependent N-hydroxy amide sulfotransferase (16, 37, 64) activities. These enzymes catalyze the conversion of certain N-hydroxy arylamines and N-hydroxy arylamides to their reactive acetic acid, serine, and sulfuric acid esters, respectively. The highly reactive and mutagenic sulfuric acid ester of N-HO-AAF is strongly implicated as an ultimate carcinogenic metabolite of AAF in rat liver (16, 17, 23, 41, 74). Similarly, reactive ester metabolites of other N-hydroxy compounds have been considered as ultimate carcinogens (48). The NADPH-dependent reduction of N-hydroxy-2-naphthylamine (52) and an S-adenosylmethionine-dependent O-methylation of N-HO-AAF (39) by hepatic cytosols have also been reported.

This communication reports the PAPS-dependent esterification of N-HO-MAB, a metabolite of the hepatocarcinogen MAB (33), and of several other carcinogenic and noncarcinogenic N-hydroxy arylamines. Some enzymatic prop-

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3 The abbreviations used are: N-HO-AAF, N-hydroxy-N-acetyl-2-aminofluorene; PAPS, 3’-phosphoadenosine 5’-phosphosulfate; AAF, N-acetyl-2-aminofluorene; PAPS, 3’-phosphoadenosine 5’-phosphosulfate; AAF, N-acetyl-2-aminofluorene; N-HO-MAB, N-hydroxy-N-methyl-4-aminoazobenzene; MAB, N-methyl-4-aminoazobenzene; Bis-Tris, bis(2-hydroxyethyl)-Tris; PAP, 3’-phosphoadenosine 5’-phosphate; N-HO-AB, N-hydroxy-4-aminoazobenzene; N-HO-EAB, N-hydroxy-N-ethyl-4-aminoazobenzene; N-(guanosin-8-yl)-MAB, N-(guanosin-8-yl)-N-methyl-4-aminoazobenzene; 3-CHsS-MAB, 3-methylmercapto-N-methyl-4-aminoazobenzene; MAB-N-sulfate, N-methyl-4-aminoazobenzene-N-sulfate; N-benzoyloxy-MAB, N-benzoyloxy-N-methyl-4-aminoazobenzene.
erties of the sulfotransferase(s) and the chemical reactivities of the metabolically formed esters were examined.

**MATERIALS AND METHODS**

**Materials.** NAD\(^+\), NADP\(^+\), glucose 6-phosphate, Bis-Tris, ATP, 3',5'-ADP(PAP), p-nitrophenol, o-iodosobenzoate, S-adenosylmethionine iodide, S-acetyl-CoA, catalase (type C-40), and serum albumin (type V) were purchased from the Sigma Chemical Company (St. Louis, Mo.). NADPH and NADH were obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.). Glucose-6-phosphate dehydrogenase (Code ZF) was purchased from Worthington Biochemicals (Freehold, N.J.). rRNA and PAPS were prepared from rat liver by the methods of Irving and Veazey (28) and Irving et al. (27), respectively. Radioactive materials were obtained as follows: [8-\(^{14}\)C]guanosine, Schwarz/Mann (Orangeburg, N.Y.); [8-\(^{14}\)C]adenosine, Amersham/Searle Corp. (Arlington Heights, Ill.); [2,6-\(^{14}\)C]uridine, Nuclear Dynamics (El Monte, Calif.); and [2,6-\(^{14}\)C]thymidine, New England Nuclear (Boston, Mass.). [G-\(^{3}\)H]-N-Hydroxy-2-naphthylamine (110 mCi/ mmole) was prepared from [G-\(^{3}\)H]-2-nitronaphthalene (Amersham/Searle custom tritiation) (75). [G-\(^{3}\)H]-N-HO-MAB was synthesized as described previously (33).

The following amine derivatives were synthesized by published procedures: N-hydroxy-N-methyl-N-benzylamine (20), N-hydroxyaniline (35), N-HO-AB (56), N-HO-MAB and N-HO-EAB (33), N-hydroxy-2-aminofluorene (40), N-HO-AAF (50), trans-N-hydroxy-4-aminostilbene (1), N-hydroxy-4-aminobiphenyl (42), N-hydroxy-1- and 2-naphthylamine (75), 2-amino-1-naphthylsulfate (13), 2,2'-azoxynaphthalene (43), MAB (46), 3-methylmercapto-4-aminobenzene and its N-methyl derivative (57), and 3-methylmercapto-4-aminobiphenyl (16). 3-Methylmercapto-N-ethyl-4-aminobenzene was synthesized by the procedure described for the synthesis of its N-methyl analog (57); after purification by chromatography on alumina and crystallization (m.p. 38\(^\circ\)), it eluted as a single peak on gas-liquid chromatography and had the expected mass spectrum (M\(^+\) = 271). Analyses for each element were within 0.2% of theory (Huffman Laboratories, Wheatridge, Colo.). 3-Methylmercapto-2-aminofluorene and 3-methylmercapto-N-acetyl-2-aminofluorene were generously supplied by Dr. T. L. Fletcher (The Fred Hutchinson Cancer Research Center, Seattle, Wash.).

Microsomal and cytosolic cell fractions were obtained by differential centrifugation according to the principles outlined by Hogeboom et al. (25). Livers were perfused in situ with 0.9% NaCl solution prior to homogenization. Adult animals were obtained as follows: CD random-bred rats and CD-1 mice, Charles River Breeding Laboratories (Wilmington, Mass.); rabbits, Sand Valley Farms (Spring Green, Wis.); guinea pigs, O'Brien Co. (Madison, Wis.); and hamsters (Con Olson Co. (Madison, Wis.). The rats, mice, and hamsters were fed Wayne Breeder Blox pellets (Allied Mills, Inc., Chicago, Ill.); the rabbits and guinea pigs were given Teklad Guinea Pig Diet (Teklad Mills, Winfield, Iowa).

**Enzyme Assays.** The enzyme-catalyzed reactions of N-HO-AAF, N-HO-MAB, and other N-hydroxy arylamines were measured by monitoring the loss of these substrates from the assay media. Each N-hydroxy compound was dissolved or diluted in absolute ethanol to a concentration of 25 mm and was added to the enzyme incubation mixture at a final concentration of 0.5 mm. The oxidative decomposition of the N-hydroxy arylamines in the reaction media was minimized by the inclusion of 0.5 mm EDTA which prevented metal-catalyzed autoxidation (31) and/or lipid peroxidation (29). This low level of EDTA stabilized the N-hydroxy derivatives even in assay media that required high concentrations of Mg\(^{2+}\). Assays that did not require oxygen were carried out in an argon atmosphere to minimize further nonenzymatic oxidation of these compounds. Unless otherwise indicated, hepatic cell fractions from adult male rats (age, 4 to 6 months) were used as enzyme sources.

Microsomal N-hydroxy amine oxidase and reductase assays were carried out as previously reported (32). Unless otherwise indicated, the assay media for measuring cytosolic S-adenosylmethionine-dependent O-methylase (39), N-hydroxy arylacetamide-dependent acetyltransferase (7), ATP-dependent seryltransferase (66), and NADPH-dependent N-hydroxy arylamine reductase (52) were used as described in the references.

Attempts to detect acetyl-CoA-dependent metabolism in N-HO-MAB were carried out in assay media that contained 100 mm Tris-HCl buffer (pH 7.3 at 37\(^\circ\)), 0.5 mm EDTA, 1 mm acetyl-CoA, 20 to 100 mm methionine, 3 mg cytosolic protein per ml, and 0.5 mm N-HO-MAB.

The assay medium that was found to be optimal for hepatic N-hydroxy arylamine sulfotransferase activity contained 100 mm Bis-Tris-HCl buffer (pH 6.2 at 37\(^\circ\)), 5 mm MgCl\(_2\), 0.5 mm EDTA, 1 mm PAPS, 0.5 to 5.0 mg cytosol protein per ml, and the specified N-hydroxy amine (0.5 mm). The assay medium that was optimal for N-HO-AAF sulfotransferase contained 100 mm Bis-Tris-HCl buffer (pH 6.6 at 37\(^\circ\)), 5 mm MgCl\(_2\), 0.5 mm EDTA, 0.65 mm PAPS, 0.2 to 1.0 mg cytosol protein per ml, and N-HO-AAF (0.5 mm). When compared directly with the assay medium described by DeBaun et al. (16), the above medium yielded 10 to 20% higher activities. L-Methionine (100 mm for N-hydroxy amine assays; 20 mm for N-HO-AAF assays), [8-\(^{14}\)C]guanosine (1 mm, 4 mCi/mmol), or rRNA (6.7 mg/ml) was routinely added to the sulfotransferase assays to trap the enzymatically generated sulfuric acid esters.

Each of the above assays was carried out in the presence or absence of the coenzymes and with intact or heat-denatured (85\(^\circ\) for 3 min) cell fractions so that coenzyme- and enzyme-dependent metabolism could be ascertained.

**Chemical Assays.** The concentrations of the N-hydroxy amines in aliquots of the incubation mixtures were determined colorimetrically as n-amyl acetate-extractable Fe\(^{3+}\)-reducing equivalents. Aliquots (0.5 ml) were agitated on a vortex mixer with 3 ml of water-saturated n-amyl acetate, and the phases were separated by centrifugation. The concentrations of the N-hydroxy secondary amines (N-HO-MAB, N-HO-EAB, N-hydroxy-N-methyl-N-benzylamine) in the amyl acetate extracts were determined by a method described for the estimation of “lipid-soluble” N-hydroxy amines (32), except that, in order to stabilize the chromatophore, 0.05 ml of 1.7 M acetic acid was added after the specified addition of EDTA.

The concentrations of N-hydroxy primary arylamines and...
phenolic amines in the amyl acetate extracts were measured by a modification of a method developed for the estimation of tocopherols as Fe³⁺-reducing equivalents (72), in which 0.2 ml of the amyl acetate extract was mixed with a solution that consisted of 0.1 ml of 0.4 M sodium acetate:0.6 M acetic acid, 0.6 ml of 95% ethanol, 0.2 ml of 4,7-diphenyl-1,10-phenanthroline [10 mg/ml in anhydrous amyl acetate: absolute ethanol (15:1)], and 0.04 ml of 0.01 M Fe(NO₃)₃-0.1 M acetic acid; after 1 min the reaction was terminated by addition of 0.04 ml of 0.02 M H₃PO₄. The absorbance at 535 nm was 39,200 (2 equivalents of Fe³⁺ reduced per equivalent of N- or o-hydroxy amine) for each of the following compounds: N-HO-AB, N-hydroxyaniline, N-hydroxy-1- and 2-naphthylamine, N-hydroxy-4-aminobiphenyl, N-hydroxy-2-aminofluorene, N-hydroxy-4-aminothionine was used to trap reactive metabolites, the methi- onyl derivatives of the aminoazo dyes were decomposed and analyzed as 3-methylmercapto-4-aminobiphenyl by gas-liquid chromatography (Ref. 33) by thin-layer chromatography in Solvents A, B, and F (33). After elution the products were analyzed by gas chromatography-mass spectrometry. The sulfonium deriva- tive of 4-aminobiphenyl was decomposed and analyzed as 3-methylmercapto-4-aminobiphenyl by gas-liquid chromatography as previously described (7).

The formation of [8-¹⁴C]-N-(guanosin-8-yl)-MAB in N-HO- MAB sulfotransferase assays that contained [8-¹⁴C]guanosine was measured by thin-layer chromatography (Ref. 38, Solvents A to D) and liquid scintillation spectrometry. Similar incubation mixtures that contained other [¹⁴C]nucleosides were chromatographed by the procedures of Poirier et al. (51). When the N-hydroxy amine sulfotransferase assays contained rRNA, the recovery and esti- mation of RNA-bound substrate were carried out according to the method of Wislocki et al. (76).

For structural identification the products formed during the PAPS-dependent esterification of N-HO-MAB were iso- lated by extraction 3 times with 2 volumes of ethyl acetate, concentration of the extract under reduced pressure, and thin-layer chromatography of the concentrate in Solvents A, B, and F (33). After elution the products were analyzed by mass spectrometry. Quantitation of these products was achieved with the use of [³⁻H]-N-HO-MAB (503 mCi/m mole) as follows. A 0.5-ml aliquot of the incubation medium was mixed with an equal volume of 95% ethanol that contained carrier dyes (0.01 M MAB, N-HO-MAB, and N-HO-AB); after centrifugation 2 /μl were analyzed as previously described (33) by thin-layer chromatography in Solvents A, B, and F and liquid scintillation spectrometry. Formaldehyde was measured by the method of Nash (49), except that prior extraction with chloroform was required to remove dye derivatives; semicarbazide (1 mm) was included in the reaction mixture to obtain quantitative recovery of the formalde- hyde.

Products from the [G⁻³⁻H]-N-hydroxy-2-naphthylamine (110 mCi/m mole) sulfotransferase reaction were isolated similarly by thin-layer chromatography on silica gel (East- man 60/60 sheets) with 1-butanol:1-propanol:0.1 M NH₄ (2:1:1) and 1-butanol:acetic acid:H₂O (10:1:1) [Solvents (a) and (m) of Boyland and Manson (11)] and with Solvents A and F (33).

**Instrumentation.** Mass spectra were obtained with a Varian CH-7 mass spectrometer (Varian Associates, Palo Alto, Calif.) equipped with a mass marker and a magnet-driven direct insertion probe (Variset Corp., Madison, Wis.). Elec- tronic spectra were measured with a Zeiss PM Q II or Beck- man DB spectrophotometer. ³H was determined in Scintisol (Isolab, Inc., Akron, Ohio) with a Packard Tri-Carb scintilla- tion spectrometer. Gas chromatography was performed with a Barber-Colman Model 10 chromatograph equipped with a 0.6 x 120-cm column packed with 3% OV-1 on Chromsorb W/HP (Pierce Chemical Co., Rockford, Ill.).

**RESULTS**

**Microsomal N-Hydroxy Arylamine Metabolism**

In view of the metabolic formation of N-hydroxy amines by the endoplasmic reticulum of liver cells, the ability of male rat liver microsomes to metabolize certain N-hydroxy amines was examined. Under the assay conditions used (cf. "Materials and Methods"), the NADH-dependent reduc- tion of N-hydroxy-N-methyl-N-benzylamine and N-hydroxy- aniline occurred at rates of 8.6 ± 0.8 and 2.1 ± 0.5 nmoles/ min/mg microsomal protein (n = 3). On the basis of analy- ses for extractable reducing equivalents, no NADH-depend- ent loss of N-HO-MAB or N-HO-EAB was observed, and these compounds were recovered quantitatively from the assay medium with incubation periods of up to 6 min. Incubation for longer times resulted in significant losses, but similar losses occurred with heat-denatured micro- somes. Similarly, 93 to 98% of the N-hydroxy derivatives of 4-aminoazobenzene, 4-aminobiphenyl, 2-aminofluorene, 4- aminostilbene, and 1- and 2-naphthylamine were recovered as extractable reducing equivalents after 6-min incubations with NADH-fortified microsomes. With fortified heat-denatured microsomes only 20 to 50% of each N-hydroxy primary arylamine was recovered under the same conditions. The much higher recoveries of these compounds from incubations with fresh microsomes may be due to a reduced pyridine nucleotide-dependent enzymatic reduction of aerobic oxidation products (nitroso derivatives?) of the N-hydroxy amines (54).

Under the standard conditions (pH 7.6 in air, cf. "Mate- rials and Methods") for measuring microsomal NADPH-de- pending N-hydroxy amine oxidase activity, the N-hydroxy aminoazo dyes oxidized rapidly (20 to 50 nmoles/min/ml of reaction mixture) even in the absence of NADPH and/or...
Cytosolic N-Hydroxy Arylamine Metabolism

In preliminary experiments with liver cytosol from male rats no coenzyme-dependent loss of N-HO-MAB or N-HO-EAB was detected (<0.1 nmole/min/mg cytosol protein) using assay media described (cf. "Materials and Methods") for N-hydroxyarylacetamide or acetyl-CoA-dependent O-acetylation, ATP-dependent O-serylization, S-adenosylmethionine-dependent O-methylilation, or NADPH-dependent reduction of certain N-hydroxy amines or N-hydroxy amides. Furthermore, the formation of a methionyl-dye adduct was not detected (<0.01 nmole/min/mg protein) in methionine-supplemented O-acetylation or O-serylization incubation mixtures. Attempts to detect these latter activities at other pH's (6.2, 6.6, 7.0), with substrate concentrations up to 0.5 mm, or with protein concentrations of 1 to 5 mg/ml were unsuccessful. However, PAPS-dependent losses of N-HO-MAB of greater than 10% occurred under the assay conditions used to demonstrate the formation of the sulfuric acid ester of N-HO-AAF (16), and 3-CH$_3$S-MAB was recovered when methionine was included in the incubation medium. Similar results were obtained with several other N-hydroxy arylamines. These experiments suggested that rat hepatic cytosol contains enzyme(s) that catalyze the PAPS-dependent esterification of N-hydroxy amines, and the properties of this enzymatic activity are described in the following section.

**N-Hydroxy Amine Sulfotransferase Activity**

**Assay Requirements and Comparisons with N-HO-AAF Sulfotransferase Activity**

The loss of N-HO-MAB and formation of 3-CH$_3$S-MAB from methionine-supplemented incubation mixtures were dependent on the presence of PAPS and cytosol (Table 1).

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Loss of N-HO-MAB</th>
<th>Formation of 3-CH$_3$S-MAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>With heat-denatured cytosol</td>
<td>5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>- PAPS</td>
<td>8</td>
<td>&lt;1</td>
</tr>
<tr>
<td>- PAPS + PAP (1 mM)</td>
<td>10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>- PAPS + ATP (1 mM)</td>
<td>12</td>
<td>&lt;1</td>
</tr>
<tr>
<td>- Methionine</td>
<td>101</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ Serum albumin (5 mg/ml)</td>
<td>101</td>
<td>59</td>
</tr>
</tbody>
</table>

* Assays were carried out as described in "Materials and Methods" with 5 to 10 different preparations of hepatic cytosol from male rats; the rates are expressed relative to that of the complete system. The mean N-HO-MAB sulfotransferase activity of the complete system was 4.7 ± 1.6 nmoles N-HO-MAB metabolized per min per mg protein (n = 10). The formation of 3-CH$_3$S-MAB by the complete system averaged 0.61 ± 0.05 nmole/min/mg cytosol protein (n = 10).

Neither ATP nor PAP could substitute for PAPS, and heat-denatured cytosol did not support the reaction. A low PAPS-independent loss of N-HO-MAB was achieved only with cytosols from 0.9% NaCl solution-perfused livers. The loss of N-HO-MAB was 5 to 10 times greater in cytosols from nonperfused livers, and variable amounts of heme or Fe$^{2+}$ in these cytosols may have promoted the rapid decomposition of the N-HO-MAB (31). Omission of methionine from the assay medium did not affect the PAPS-dependent loss of N-HO-MAB, but addition of methionine was essential to the formation of a sulfonium-dye conjugate. Protein (added serum albumin) apparently competed with methionine for the reactive ester.

Experiments with N-HO-MAB and other N-hydroxy arylamines indicated that the optimal conditions for N-hydroxy amine sulfotransferase activity differed from those for sulfotransferase activity for N-HO-AAF or 3-hydroxyxanthine (16, 64); the major differences were the optimal pH and the protein and PAPS concentrations. The optimal assay conditions for sulfotransferase activity for N-HO-MAB and N-HO-AAF (cf. "Materials and Methods") were defined after testing different methods of rat hepatic cytosol preparation (with or without liver perfusion, various homogenization media, gel filtration of the cytosol), incubation times, pH, buffer compositions, and the concentrations of Mg$^{2+}$, EDTA, PAPS, substrate, substrate solvent (ethanol, acetone, or dimethyl sulfoxide), and cytosol protein. Under the optimal assay conditions the PAPS-dependent loss of N-HO-MAB and the formation of 3-(methion-S-yl)-N-methyl-4-aminoazobenzene (determined as 3-CH$_3$S-MAB) were 1st order with respect to protein concentration up to 5 mg/ml and linear with time for 10 to 15 min. The loss of N-HO-AAF and the formation of 1- and 3-(methion-S-yl)-N-acetyl-2-aminofluorene (as o-methylmercapto-N-acetyl-2-aminofluorene) were 1st order with protein concentration up to 1 mg/ml and linear with time for 30 to 60 min. Inhibition by PAPS occurred at concentrations above 1.0 and 0.65 mm, respectively, for N-HO-MAB and N-HO-AAF sulfotransferase activities.

The pH optima for the cytosol-catalyzed PAPS-dependent losses of N-HO-MAB and N-HO-AAF, as well as for the formation of their sulfonium derivatives, were approximately 6.2 and 6.6, respectively (Chart 1). The pH versus
rate profile for N-HO-AAF sulfotransferase activity is similar to that described by DeBaun et al. (16).

Previous studies on the sulfonation of phenols, amines, and steroids have demonstrated that several cytosolic proteins catalyze PAPS-dependent sulfonation reactions (19). These sulfotransferases have multiple and overlapping substrate affinities and differ in their pH optimum and sensitivity to inhibitors and activators. A "phenol" sulfotransferase that reportedly esterifies only phenols is unaffected by added Mg$^{2+}$, has optimal activity at pH 5.8, and, depending on the thiol content of the enzyme preparation, is activated or inhibited by o-iodosobenzoate (3, 4, 15, 45). Another sulfotransferase that acts on androgens, cholesterol, 2-naphthylamine, L-nitrophenol, and 3-tirosine methyl ester is stimulated by Mg$^{2+}$, has optimal activity at pH 7.0 to 7.5, and is inhibited by o-iodosobenzoate (3, 5, 15). A 3rd sulfotransferase that acts on estrogens, 2-naphthylamine, and L-nitrophenol is stimulated by Mg$^{2+}$; it has optimal activity at pH 6.2 for estrogens and 2-naphthylamine and at pH 7.0 for L-nitrophenol (3, 19). Sulfonation of 2-naphthylamine is stimulated by addition of steroids while steroid sulfonation is inhibited by added 2-naphthylamine (3, 55). Another steroid sulfotransferase is thought to catalyze the esterification of deoxycorticosterone, but no further characterization has been made (3). An o-iodosobenzoate-sensitive sulfotransferase with activity toward L-tyrosine methyl ester, tyramine, and L-nitrophenol has also been described (5, 44).

As an aid in evaluating the roles of the various sulfotransferases in the esterification of N-HO-AAF and N-HO-MAB, the effects of several inhibitors and activators on these reactions were investigated. The assays were carried out either under optimal conditions for N-HO-MAB (1 mM PAPS, pH 6.2) and for N-HO-AAF (0.65 mM PAPS, pH 6.6) or under intermediate conditions (0.65 mM PAPS, pH 6.4), so that both activities could be measured on a comparable basis (Table 2). At pH 6.4, omission of Mg$^{2+}$ decreased the activity toward both N-HO-MAB and N-HO-AAF by 10 to 20%. At pH 7.0, the effect on N-HO-AAF activity was more pronounced; the rate in the absence of added Mg$^{2+}$ was only 50 to 60% of the control level. These differences may reflect the catalytic participation of several sulfotransferases and/or the inhibitory effect of Mg$^{2+}$ on PAPS-sulfohydrolase (18). o-Iodosobenzoate inhibited both N-HO-MAB and N-HO-AAF sulfotransferase activity, but it was a much more effective inhibitor of the latter activity. Both 2-naphthylamine and L-nitrophenol inhibited the esterification of N-HO-MAB and N-HO-AAF. Dehydroepiandrosterone, estrone, and deoxycorticosterone increased sulfotransferase activity for N-HO-AAF by 20 to 40%, while only estrone and deoxycorticosterone appeared to increase sulfotransferase activity for N-HO-MAB. L-Tyrosine methyl ester did not significantly alter either N-HO-MAB or N-HO-AAF sulfotransferase activity. The addition of N-HO-AAF to assays for N-HO-MAB sulfotransferase activity caused a 40% stimulation, while N-HO-MAB was strongly inhibitory to N-HO-AAF sulfotransferase activity. Although several sulfotransferases may catalyze the esterification of both N-HO-MAB and N-HO-AAF, the activity for N-HO-MAB appears to be similar to that described for

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>3-CH$_3$S-MAB</th>
<th>2-CH$_3$S-MAB</th>
<th>1-CH$_3$S-MAB</th>
<th>2-H$_2$S-MAB</th>
<th>1-H$_2$S-MAB</th>
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<td>86</td>
<td>86</td>
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<td>+ o-iodosobenzoate (0.25 mM)</td>
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<td>+ 2-Naphthylamine (2.0 mM)</td>
<td>23</td>
<td>33</td>
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<tr>
<td>+ p-Nitrophenol (1.0 mM)</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
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<tr>
<td>+ Dehydroepiandrosterone (0.1 mM)</td>
<td>98</td>
<td>128</td>
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<td>+ Estrone (0.1 mM)</td>
<td>113</td>
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<tr>
<td>+ Deoxycorticosterone (0.1 mM)</td>
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<td>+ L-Tyrosine methyl ester (3 mM)</td>
<td>104</td>
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<td>+ N-HO-AAF (0.5 mM)</td>
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<td>+ N-HO-MAB (0.5 mM)</td>
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<td></td>
<td>13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Effects of various agents on rat hepatic N-HO-MAB and N-HO-AAF sulfotransferase activities

* The incubation medium was a compromise between the optimal systems for N-HO-MAB and N-HO-AAF and contained 100 mM Bis-Tris-HCl buffer (pH 6.4 at 37°C), 100 mM methionine, 5 mM MgCl$_2$, 0.5 mM EDTA, 0.65 mM PAPS, rat liver cytosol protein (1 mg/ml), and 0.5 mM N-HO-MAB or N-HO-AAF. Incubation time was 15 min.

* Assays were carried out as described in "Materials and Methods" using 4 to 6 different cytosol preparations; the rates are expressed relative to the control. The mean rates of methylmercaptoarylamine formation in the complete system were 0.31 ± 0.03 and 6.1 ± 0.5 nmoles/min/mg (n = 6) for N-HO-MAB and N-HO-AAF, respectively. Similar relative rates were obtained under the various reaction conditions by measuring the PAPS-dependent loss of N-HO-MAB or N-HO-AAF.

"phenol" sulfotransferase, while the activity for N-HO-AAF exhibits properties similar to those described for both phenol and steroid sulfotransferases.

Reactivity of MAB-N-sulfate

A relatively high concentration of methionine (100 mM) was required to trap appreciable amounts of the sulfuric acid ester of N-HO-MAB, and under these conditions the formation of a sulfonium dye-conjugate accounted for only 5 to 15% of the PAPS-dependent loss of N-HO-MAB (Chart 2). With N-HO-AAF as substrate 20 mM methionine trapped 65 to 85% of the reactive ester as a methionyl derivative.

Addition of [8-14C]guanosine (optimal concentration, 0.8 to 1.2 mM) to the sulfotransferase incubation medium yielded a [8-14C]guanosinyl adduct that accounted for 6 to 12% of the N-HO-MAB metabolized. The adduct was identified as [8-14C]-N-(guanosin-8-yl)-MAB on the basis of its chromatographic properties in 4 thin-layer systems (38). Similar sulfotransferase incubation mixtures that contained 1 mM [14C]adenosine, [14C]cytidine, [14C]uridine, or [14C]-thymidine yielded no significant amounts of nucleoside-dye adducts (<0.5% of the N-HO-MAB metabolized). This result was consistent with the lack of detectable reactivity of these nucleosides with the synthetic ester N-benzoyloxy-MAB (51). The sulfuric acid ester of N-HO-MAB also reacted with added rRNA (6.7 mg/ml), but only 1 to 3% of the N-hydroxyamine metabolized was recovered as an rRNA adduct.
Tissue, Sex, and Species Distribution

Of the extrahepatic tissues studied only the cytosol fractions of rat kidney and small intestine contained detectable sulfotransferase activity for N-HO-MAB (Table 3). These activities are no more than 20% of that found in hepatic cytosol, but a comparable level of sulfotransferase activity for N-HO-AAF was not detected in rat kidney or small intestine (16). No activity for N-HO-MAB was detected in rat muscle or lung cytosols.

The highest hepatic sulfotransferase activity for N-HO-MAB was found in male rats. Female rat liver and the livers from males of the other species studied, except the hamster, had at least 25% of the activity of male rat liver (Table 4). N-HO-AAF sulfotransferase activity has been detected only in rat and rabbit liver cytosols (16).

Young adult animals were used in these comparative studies. In young rats hepatic N-HO-MAB sulfotransferase activity was consistently lower than in older rats (cf. Tables 1 and 5). Thus, the lower N-HO-MAB activities shown in Tables 3 and 4 reflect this age variation.

Substrate Specificity

N-HO-EAB, N-HO-AB, N-hydroxy-1- and 2-naphthylamine, and N-hydroxy-4-aminobiphenyl also appeared to be converted to reactive esters by rat hepatic cytosol and PAPS (Table 5). The PAPS-dependent metabolism of each of these N-hydroxy amines was optimal under the assay conditions for N-HO-MAB sulfotransferase rather than those for N-HO-AAF esterification. In the absence of PAPS, 80 to

Table 3

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PAPS-dependent loss of N-HO-MAB (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2.7 ± 0.9 *</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.5 ± 0.3 *</td>
</tr>
<tr>
<td>Small intestine mucosa</td>
<td>0.5 ± 0.3 *</td>
</tr>
<tr>
<td>Muscle</td>
<td>&lt;0.2 *</td>
</tr>
<tr>
<td>Lung</td>
<td>&lt;0.2 *</td>
</tr>
</tbody>
</table>

* Assays were carried out with the cytosol fraction of each tissue homogenate, and the values reported represent determinations on tissues from 3 animals.

* For each tissue at least 2 reactions were also carried out in the presence of methionine. In each case the amount of 3-CH₃-S-MAB that could be isolated at the end of the incubation period was equivalent to 5 to 10% of the PAPS-dependent loss of N-HO-MAB.

* Mean ± S.D.

* These values were judged to be the lower limit of detection.

Table 4

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>No. of animals</th>
<th>PAPS-dependent loss of N-HO-MAB (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>M</td>
<td>10</td>
<td>3.6 ± 1.8 *</td>
</tr>
<tr>
<td>Rat</td>
<td>F</td>
<td>5</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>M</td>
<td>3</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>M</td>
<td>3</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Mouse</td>
<td>M</td>
<td>3</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Hamster</td>
<td>M</td>
<td>3</td>
<td>0.2 ± 0.03</td>
</tr>
</tbody>
</table>

* See "Materials and Methods" for assay procedure. For the livers from each species at least 2 reactions were carried out in the presence of methionine. In each case the amount of 3-CH₃-S-MAB that could be isolated at the end of the incubation period was equivalent to 5 to 10% of the PAPS-dependent loss of N-HO-MAB.

* Mean ± S.D.

Table 5

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PAPS-dependent loss of substrate (nmol/min/mg protein)</th>
<th>% isolable as methylmercaptoarylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-HO-MAB</td>
<td>4.8 ± 1.8 *</td>
<td>5-20</td>
</tr>
<tr>
<td>N-HO-EAB</td>
<td>2.2 ± 0.6</td>
<td>8-12</td>
</tr>
<tr>
<td>N-HO-AB</td>
<td>1.9 ± 0.6</td>
<td>1-3</td>
</tr>
<tr>
<td>N-Hydroxy-1-naphthylamine</td>
<td>2.4 ± 1.0</td>
<td>- *</td>
</tr>
<tr>
<td>N-Hydroxy-2-naphthylamine</td>
<td>1.4 ± 0.3</td>
<td>- *</td>
</tr>
<tr>
<td>N-Hydroxy-4-aminobiphenyl</td>
<td>1.1 ± 0.4</td>
<td>25-40</td>
</tr>
<tr>
<td>trans-N-Hydroxy-4-aminobistilene, N-hydroxy-2-aminofluorenone, N-hydroxylaniline, and N-hydroxy-N-methyl-N-benzyllamine</td>
<td>&lt;0.1</td>
<td>- *</td>
</tr>
</tbody>
</table>

* See "Materials and Methods" for assay procedure. For the livers from each species at least 2 reactions were carried out in the presence of methionine. In each case the amount of 3-CH₃-S-MAB that could be isolated at the end of the incubation period was equivalent to 5 to 10% of the PAPS-dependent loss of N-HO-MAB.

* Mean ± S.D. for analyses of 5 to 10 different cytosol preparations. The wide standard deviations reflect the variations in activity from one liver preparation to another.

* Not studied.

* The trapping efficiencies differed between liver cytosols, possibly because of variations in the amounts of endogenous nucleophiles.

* Assays were carried out as described in "Materials and Methods."
100% of each N-hydroxy amine added could be recovered after the 10-min incubation period; the low PAPS-independent losses were presumed to be nonenzymatic. When methionine or rRNA was added to the complete assay media, methionyl or rRNA adducts were formed.

Products Formed in the Sulfotransferase Incubation Medium

From N-HO-MAB. Incubation of N-HO-MAB in the sulfotransferase assay in the absence of added guanosine, methionine, or RNA yielded the following products (expressed as the percentage of the N-HO-MAB metabolized): MAB (50 to 60%), N-HO-AB (25 to 35%), formaldehyde (20 to 30%), and 2 unidentified products (15 to 25%). MAB and N-HO-AB were identified by thin-layer chromatography, their UV absorption maxima in 95% ethanol (402 and 375 nm, respectively), and their mass spectra (M+ = 211 and 213, respectively). Formaldehyde was characterized spectrally as the 2,4-pentanedione-ammonia adduct (λmax = 412 nm). The unidentified products exhibited absorption maxima at 390 to 400 nm in 95% ethanol and at 520 to 530 nm in 40% ethanol:0.5 M HCl; these products had low volatilities and did not yield molecular ions on electron impact mass spectrometry. These properties are consistent with those expected for a dinitrone or a nitrone-N-hydroxy amine addition product. All of the above reaction products were also obtained in assays supplemented with methionine or guanosine, but the yields were proportionately decreased.

A reaction mechanism that is consistent with the above results is presented in Chart 3. In view of the great lability of the N-sulfate of N-HO-AAF and some other esters of N-hydroxy metabolites (7, 41, 48, 51, 60), the apparent initial product from N-HO-MAB, MAB-N-sulfate, is presumed to be rapidly converted to a reactive nitrenium ion. This ion could then bind to cellular nucleophiles (cf. Ref. 59). However, unlike N-hydroxy amides (e.g., N-HO-AAF), N-hydroxy amines such as N-HO-MAB are strong reducing agents. The latter property may account for the apparent reduction of the aminoazo nitrenium ion to MAB and the concomitant formation of oxidation products (N-HO-AB and nitrone-derived adducts) of N-HO-MAB. Reduction of acrylaryl nitrenium ions by several reducing agents has been demonstrated (61, 65). This reaction sequence may account for the low yields of arylamine-nucleophile adducts formed from N-hydroxy amines, as compared to the high yields of such adducts obtained from esters of N-HO-AAF.

Further support for this mechanism was obtained by incubation of 0.5 μmole of N-benzoyloxy-MAB with 0.5 μmole of N-HO-MAB under the sulfotransferase assay conditions, except for the omission of PAPS and cytosol. Both of the added dyes were consumed within 10 min, and 0.16 μmole of MAB, 0.28 μmole of N-HO-AB, 0.14 μmole of formaldehyde, and 5 to 6 other unidentified dye-containing products were obtained. Incubation of N-HO-MAB in the absence of N-benzoyloxy-MAB under these conditions yielded only the starting material, while incubation of N-benzoyloxy-MAB alone yielded several of the above unidentified dye products.

From N-Hydroxy-2-naphthylamine. A similar reaction sequence appeared to occur when [3H]-N-hydroxy-2-naphthylamine was incubated with PAPS and hepatic cytosol (Chart 4), but the identification of the naphthalene derivatives was hampered by the relatively low rate of esterification of this N-hydroxy amine. The tentative identifications of the products are based on their chromatographic behaviors in 2 thin-layer systems and the characteristic fluorescence of each product under 254 nm light (10). The formation of each product was dependent on the presence of PAPS in the incubation mixture. 2-Amino-1-naphthyl sulfate accounted for 30 to 35% of the substrate metabolized. 2-Naphthyla-
mine and 2,2'-azoxynaphthelene, which were only partially resolved by chromatography, accounted for another 35 to 40%. While the 5 to 10% yield of 2-amino-1-naphthol was dependent on PAPS, 2-amino-1-naphthol was converted to 2-amino-1-naphthyl sulfate in the sulfotransferase reaction medium at 5 to 10 times the rate of conversion of N-hydroxy-2-naphthylamine to this product. The reaction scheme, while only tentative, allows for both sulfamate and ester formation and is consistent with the reported chemical properties of N- and O-sulfonates of N-hydroxy arylamines (12-14).

Conversion of MAB to N-(Guanosin-8-yl)-MAB by the 10,000 x g Supernatant of Rat Liver

By appropriate modifications of the MAB N-oxidase (33) and N-HO-MAB sulfotransferase assay procedures, it was possible to demonstrate the conversion of MAB to a nucleoside adduct on incubation with the 10,000 x g supernatant fraction of rat liver. For this purpose [3H]MAB and guanosine were used as substrate and trapping agent, respectively. Demonstration of this overall reaction required the selection of an intermediate pH and elimination of potassium phosphate buffer, which inhibited sulfotransferase activity, and Tris-HCl buffer, which inhibited N-oxidation. Methionine (100 mm) could not be used as the trapping agent, since it completely inhibited the N-oxidase. PAPS was also somewhat inhibitory (40%) to the N-oxidase. The incubation medium selected for demonstration of the reaction contained 100 mm Bis-Tris-HCl buffer (pH 7.0 at 37°C), 1 mm NADH-generating system (33), 1 mM guanosine, 5 mg thionine (100 raM) could not be used as the trapping agent, somewhat inhibitory (40%) to the N-oxidase. The incubation medium selected for demonstration of the reaction contained 100 mm Bis-Tris-HCl buffer (pH 7.0 at 37°C), 1 mm NaN₃, 5 mm MgCl₂, 0.5 mm EDTA, 1 ppm PAPS, an NADPH- and NADH-generating system (33), 1 mm guanosine, 5 mg protein from 10,000 x g supernatant per ml, and 0.5 mm [3H]MAB (1.5 Ci/m mole). The isolation, separation, and estimation of [3H]-N-(guanosin-8-yl)-MAB were carried out as described above for the 14C-labeled derivative. Under these conditions the formation of [3H]-N-(guanosin-8-yl)-MAB from [3H]MAB was demonstrated and shown to be enhanced by the addition of both PAPS and a NADPH, NADH-generating system (Chart 5).

**DISCUSSION**

The hepatocarcinogen MAB is N-hydroxylated by a mixed-function amine oxidase in the hepatic endoplasmic reticulum (33). As shown in this report the product of this oxidation, N-HO-MAB, is sulfonated by 1 or more sulfotransferases in the hepatic cytosol in a PAPS-dependent reaction to form a reactive electrophile, presumably the ester MAB-N-sulfate. This electrophile forms products of known structure with nucleophiles such as methionine and guanosine that are identical to products derived from the protein- and nucleic acid-bound dyes formed in vivo in the livers of rats fed MAB (36, 58). MAB-N-sulfate is thus a possible ultimate carcinogenic metabolite of MAB. Evidence supporting this concept has come from the recent observations of Blunk and Crowther (8) that the hepatocarcinogenicity of the closely related dye, 3'-methyl-N,N-di-methyl-4-aminoazobenzene, is greatly enhanced in the rat by dietary administration of a high level of sodium sulfate. These data on the metabolism and carcinogenicity of MAB thus closely parallel data previously obtained for the metabolism and carcinogenicity of AAF in the rat liver (16, 17, 23, 48, 69, 74), although the N-oxidase and sulfotransferase activities for MAB and N-HO-MAB, respectively, differ from those for AAF and N-HO-AAF. The microsomal N-oxidase for MAB does not depend on cytochrome P-450 and presumably is a flavoprotein amine oxidase similar to that described by Ziegler et al. (33, 78). The microsomal oxidase for AAF involves cytochrome P-450 (69). Similarly, as shown in the present report (cf. "Results"), the hepatic sulfotransferase activities for N-HO-AAF and N-HO-MAB appear to be different. The former activity appears to have properties noted for both phenol and steroid sulfotransferases, while the latter activity appears to be more similar to that of the phenol sulfotransferase.

The inability to detect either O-acetylation or O-erythylation of N-HO-MAB, as has been demonstrated for other carcinogenic N-hydroxy arylamines (6, 7, 36, 66, 67), emphasizes the probable importance of O-sulfonation in the metabolism of this N-hydroxy dye. The failure of hepatic cytosols (NADPH-dependent) and of hepatic microsomes (NADH-dependent) to catalyze the reduction of certain N-hydroxy arylamines further conserves these substrates for this metabolic pathway.

The tissue, sex, and species distributions of microsomal N-oxidase activity for MAB (33) and cytosolic sulfotransferase activity for N-HO-MAB (Tables 3 and 4) are consistent with the observation that MAB and related aminoazo dyes are strongly carcinogenic only in the liver of the male rat (47). Likewise, the low hepatocarcinogenicities of EAB and AB (2, 47, 68), 1-naphthylamine (Ref. 24, pp. 100-101; Ref. 30, p. 168; Ref. 62, p. 183; Ref. 63, p. 126; Ref. 70, p. 559; Ref. 71, pp. 271-272), 2-naphthylamine (Ref. 24, pp. 101-103; Ref. 30, p. 169-170; Ref. 62, pp. 183-185; Ref. 63, pp. 127-128; Ref. 70, pp. 559-564; Ref. 71, p. 272), and 4-aminoazobenzene (Ref. 30, p. 104; Ref. 62, p. 102; Ref. 63, p. 71; Ref. 70, pp. 375-376; Ref. 71, p. 200) may result in part from the lower rates of O-sulfonation of the N-hydroxy metabolites of these amines in the rat liver as compared to those noted for N-HO-MAB (Table 5).

**Chart 5.** N-Oxidation of MAB and O-estification of N-HO-MAB by the 10,000 x g supernatant of rat liver.
N-[(P-Phenylazo)phenyl]nitrene also deserves consideration as a possible ultimate carcinogen of N-HO-MAB. As noted previously (33), N-HO-MAB is easily oxidized aerobically to a reactive derivative, presumably a nitrene, that can form adducts in anhydrous media with carbon-carbon and carbon-nitrogen double bonds in a variety of tissue constituents. From data presented in this report, this nitrene also appears to be formed during the oxidation of N-HO-MAB by metabolically formed MAB-N-sulfate to yield products including MAB, N-HO-AB, and formaldehyde. The latter 2 products may arise by hydrolysis of N-[(P-phenylazo)phenyl]nitrene, an initial product in the oxidation-reduction reaction. A reaction between N-benzoylxy-MAB and N-HO-MAB occurred in the absence of cytosol and PAPS and yielded similar products. In a previous report (33) it was noted that N-HO-EAB did not form a reactive nitrene upon aerobic oxidation. Thus, these observations are consistent with the earlier findings that an N-methyl group was required for the formation of high levels of protein-bound dye in vivo and for strong hepatocarcinogenicity by amionoazo dyes in the rat (2, 47, 68).

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Hepatic Metabolism of N-Hydroxy- N-methyl-4-aminoazobenzene and Other N-Hydroxy Arylamines to Reactive Sulfuric Acid Esters

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*Cancer Res* 1976;36:2350-2359.

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