Metabolism of Arylhydrazines by Mouse Liver Mixed-Function Oxidases in Vitro

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The metabolism of a group of arylhydrazines and arylhydrazides by mouse liver mixed-function oxidases was studied in vitro. The arylhydrazines, 4-methylphenylhydrazine hydrochloride and the N-acetyl derivatives of 4-methylphenyl- and 4-(hydroxymethyl)phenylhydrazine, were all readily metabolized. Their metabolism was inhibited by metyrapone and carbon monoxide. Agaritine, the arylhydrazide [N^{β}-[(+)-γ-glutamyl]-4-(hydroxymethyl)phenylhydrazine] (A), was poorly metabolized. The small amount of metabolism of A was not inhibited by metyrapone or carbon monoxide, suggesting that the breakdown represented nonspecific decomposition, not metabolism.

The hydrazide agaritine (A) or N^{β}-[(+)-γ-glutamyl]-4-(hydroxymethyl)phenylhydrazine is found in relatively large amounts [ranging from 0.04% w/w (Levenberg, 1960, 1961) to 0.3% w/w (Ross et al., 1982)] in the cultivated mushroom *Agaricus bisporus*. A can be metabolized in the mushroom (Levenberg, 1961) to glutamate and 4-(hydroxymethyl)phenylhydrazine and the latter further metabolized to the 4-(hydroxymethyl)benzenediazonium ion (Gigliotti and Levenberg, 1964). While A is not carcinogenic, the closely related homologue *N'*-acetyl-4-(hydroxymethyl)phenylhydrazine (HMPH) and 4-methylphenylhydrazine (MPH), a reduction product of 4-(hydroxymethyl)phenylhydrazine, are both carcinogenic in Swiss mice (Toth et al., 1977, 1978). These carcinogenicity findings motivated our investigation into their mode of activation. Our initial study describes their metabolic conversion to diazonium ions by a model system—mouse liver cytochrome P-450 mixed-function oxidase (MFO)—and is preliminary to the investigation of the interaction of diazonium ions with cellular constituents such as DNA.

MATERIALS AND METHODS

Animals. Eight-week-old male Swiss mice from the Eppley Institute colony were used. Food (Wayne Lab Blox, Allied Mills, Chicago, IL) and water were freely given.

Chemicals. 2-Naphthol was obtained from Aldrich Chemical Co. (Milwaukee, WI). MPH was purchased from Eastman Organic Chmicals (Rochester, NY) and recrystallized from ethanol. HMPH, *N'*-acetyl-4-methylphenylhydrazine (AMPH) and A were prepared at the institute (Toth et al., 1978; Wallcave et al., 1979). The 2-hydroxy-1-naphthalene azo derivatives were prepared by the method of Ross et al. (1982). The cytochrome P-450 inhibitor metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was obtained from Sigma Chemical Co. (St. Louis, MO).

Isolation of Mouse Liver Microsomal Preparation. Mice were lightly anesthetized with ether and decapitated to exsanguinate the livers, which were rapidly excised and washed in ice-cold saline. The microsomal fraction (105000*g* sediment) was isolated by the differential centrifugation method of Fang and Strobel (1977).

Incubation Conditions. The incubations were performed in 0.1 M phosphate buffer (pH 7.4) supplemented with 0.125 M sucrose and 5.5 mM magnesium chloride. Phosphate buffer was used instead of the more conventional Tris buffer, as it had the greater buffering capacity needed to deal with the changes in pH produced by the acidic hydrazines. Microsomal protein was added to a final concentration of 1 mg/mL. A NADPH-generating system was used and consisted of glucose 6-phosphate (5.0 mM), glucose-6-phosphate dehydrogenase (type VII) (50 units), and nicotinamide adenine dinucleotide phosphate (2.5 mM). The hydrazines were added at final concentrations up to 1.0 mM. The reaction volume was 2.5 mL. Reactions were performed in 25-mL Erlenmeyer flasks for up to 10 min and terminated by adding 5 mL of 2-naphthol (2% w/v) in methanol. The terminated reaction mixture was allowed to stand overnight to facilitate color development, after which the mixture was centrifuged to remove microsomal protein and to clarify the supernatant. The supernatant was extracted twice with 2 volumes of diethyl ether, and the extracts were combined and taken to dryness in a stream of nitrogen. The residue was taken up in 1.0 mL of spectroscopic-grade ethanol for chromatographic analysis.

The wavelengths at which maximum absorption occurred were determined on a Beckman Acta CIII spectrophotometer using methanolic solutions of authentic adducts. For the *p*-tolyl compound, λ_{max} = 425 nm and, for the *p*-(hydroxymethyl)phenyl compound, λ_{max} = 475 nm.

Mass Spectral Analysis. To obtain sufficient adduct for GC–mass spectral analysis, the products of several in vitro incubations were combined. After color development the reaction mixture was extracted with 2 volumes of diethyl ether. The ether extracts were taken to dryness in a nitrogen stream, and the residue was taken up in a small volume for thin-layer chromatography, which was performed on precoated 20 × 20 cm silica gel plates (1 mm thick). The methanolic extracts were applied as bands and authentic standards as spots at either end of each band, all of which were developed in ether–hexand (22:3). The band that corresponded to the standard was scraped from the plate while damp, packed into a small column, and eluted with methanol. UV spectral analysis indicated the presence of 2-naphthol in the eluate. The eluate was then treated with 5 N NaOH to remove the 2-naphthol and extracted with 2 volumes of diethyl ether. Ether extracts were pooled, dried overnight with Na_2SO_4, and taken to near dryness in a stream of nitrogen and further purified by high-pressure liquid chromatography, which was performed on a Waters M-6000 apparatus using an ODS-Partisil reverse-phase column. The solvent was methanol–water (4:1 v/v) at a flow rate of 1.0 mL/min (1150 psi). The retention times of 1-[(2-hydroxy-1-naphthalene)-azo]-4-methylbenzene and 1-[(2-hydroxy-1-napthalene)-azo]-4-(hydroxymethyl)benzene were 6.5 and 8.3 min, respectively.

The purified azo compounds were then subjected to GC–mass spectra analysis on a Varian 3700 apparatus with a 6-ft glass column packed with 4% SE-30 on Chromasorb W. The flow rate was 15 cm^3 of helium/min, and the

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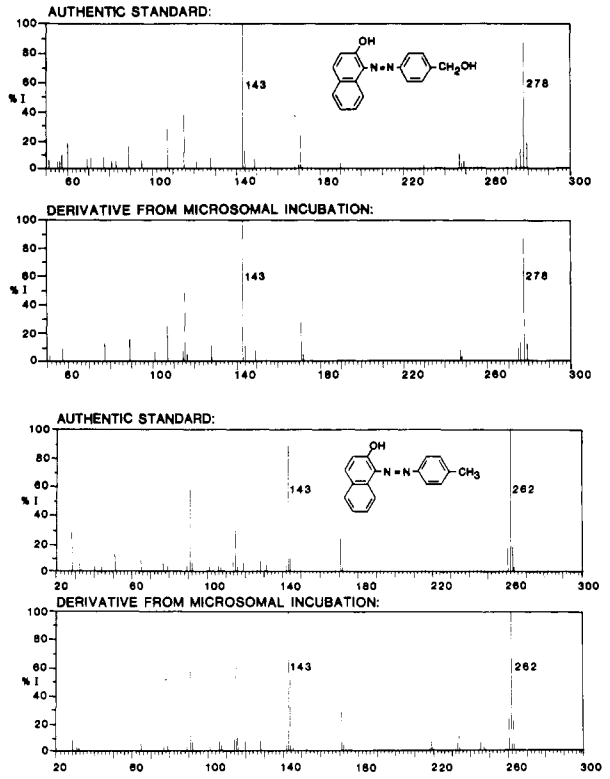

Figure 1. Mass spectra of 1-[(2-hydroxy-1-naphthalene)azo]-4-(hydroxymethyl)benzene and 1-[(2-hydroxy-1-naphthalene)-azo]-4-methylbenzene.

Table I. Metabolism of Arylhydrazines by Mouse Liver Microsomal MFO

substrate concn, μmol/2.5 mL	nmol of β-naphthol adduct/mg of microsomal protein[a]			
	A	MPH	AMPH	HMPH
0.025	2.1 ± 0.1	9.4 ± 0.8	0.9 + 0.2	
0.125	3.6 ± 0.6	21.9 ± 2.9	4.8 ± 0.2	5.7 ± 1.0
0.25	5.3 ± 0.6	31.4 ± 12.2	8.5 ± 1.9	13.4 ± 1.7
1.25	6.7 ± 1.9	66.9 ± 9.9	13.9 ± 1.7	21.2 ± 6.2
2.5	4.7 + 1.1	72.7 ± 4.5	26.2 + 0.3	22.3 + 1.6

[a] Mean ± SD; two measurements per point.

column temperature was 240 °C. The effluent was monitored with a flame ionization detector attached to an A.E.I. MS 902. The electron impact mass spectra of the authentic and isolated compounds were virtually identical (Figure 1).

RESULTS

In a preliminary experiment, a 5-min incubation was found to give optimum products. For this study a concentration of 500 mM was arbitrarily chosen. In all subsequent work, the incubations proceeded for 5 min.

The maximum substrate concentration was also determined. Substrate concentrations of 0.025, 0.125, 0.25, 1.25, and 2.5 mol/2.5 mL (10, 50, 100, 500, and 1000 mM, respectively) were used, and the amounts of products formed are given in Table I. A was poorly metabolized by the MFO, and its metabolism by mouse liver S9 fraction (9000g supernatant) was compared with that produced by the microsomal preparation. In this experiment 6.1 ± 1.1 nmol of the 2-naphthol adduct was produced by the microsomal preparation, compared with 10.7 ± 0.6 nmol by the S9 preparation.

Table II. Effect of Inhibitors of MFO on the in Vitro Metabolism of Arylhydrazines[a]

inhibitor	in nmol of β-naphthol adduct/mg of microsomal protein[b]			
	A	MPH	AMPH	HMPH
none	6.1 ± 0.2	72.7 ± 4.5	26.2 ± 0.3	27.5 ± 3.3
metyrapone[c]	6.1 ± 0.3	27.1 ± 1.1	0.4 ± 0.1	8.3 ± 1.1
carbon monoxide[d]	3.6 ± 1.2	8.7 ± 0.3	1.0 ± 0.2	1.4 ± 0.7
boiled microsomes	2.9 ± 0.7		0.2 ± 0.1	

[a] 2.5 μmol/2.5 mL. [b] mean ± SD; two measurements each. [c] 1.0 mm. [d] The flasks were gassed with carbon monoxide for the duration of the incubation.

The effects of MFO inhibitors, metyrapone and carbon monoxide, on the metabolism of the arylhydrazines are shown in Table II. Both inhibitors significantly reduced the metabolism of MPH, AMPH, and HMPH but were without effect on the metabolism of A.

DISCUSSION

The arylhydrazines and hydrazides used in this study were all capable of metabolism by mouse liver cytochrome P-450 mediated MFO. A, however, was poorly metabolized, and its metabolism was not affected by the inhibitors of cytochrome P-450. The diazonium ions liberated by the mettabolism were clearly sufficiently stable to survive the duration of the incubation and then to react with the 2-naphthol. This suggests that they would be sufficiently stable to interact with DNA in vivo and hence to generate the sequence of events believed involved in carcinogenesis. Without the availability of radiolabeled hydrazines, the extent of interaction of the diazonium ions and the microsomal proteins is not known.

A greater metabolism of A was produced by the S9 fraction than by the microsomal fraction. This is no doubt due to the presence in the cytoplasm of an enzyme that would remove the glutamic acid moiety from A, making the subsequent molecule more amenable to the MFO.

Registry No. MPH, 539-44-6; AMPH, 61700-79-6; HMPH, 65734-38-5; A, 2757-90-6; mixed function oxidase, 9040-60-2.

LITERATURE CITED

Fang, W. F.; Strobel, H. W. *Arch. Biochem. Biophys.* **1977**, *186*, 128.
Gigliotti, H. J.; Levenberg, B. *J. Biol. Chem.* **1964**, *239*, 2274.
Levenberg, B. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1960**, *19*, 6.
Levenberg, B. *J. Am. Chem. Soc.* **1961**, *83*, 507.
Ross, A. E.; Nagel, D. L.; Toth, B. *J. Agric. Food. Chem.* **1982**, *30*, 521.
Toth, B.; Nagel, D; Patil, K.; Erickson, J.; Antonson, K. *Cancer Res.* **1978**, *38*, 117.
Toth, B.; Tompa, A.; Patil, K. *Z. Krebsforsch. Klin. Onkol.* **1977**, *89*, 245.
Wallcave, L.; Nagel, D.; Raha, C. R.; Hwan-Soo, J.; Bronczyk, S.; Kupper, R.; Toth, B. *J. Org. Chem.* **1979**, *44*, 3752.

Received for review May 25, 1984. Accepted November 29, 1984. Supported by Grant RO1-CA31611 from the National Cancer Institute.