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The Role of Cytochrome P-450 in N-Hydroxylaiton of 2-Acetylaminofluorene

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SUMMARY

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The role of cytochrome P-450 mixed-function oxidases in the *N*-oxidation of 2-acetylaminofluorene has been studied. *N*-Hydroxylation of 2-acetylaminofluorene in liver microsomes from mice or hamsters was inhibited by a carbon monoxide–oxygen (90:10) atmosphere. Prior treatment of mice with cobaltous chloride for 3 days decreased both the amount of cytochrome P-450 and the rate of *N*-hydroxylation of 2-acetylaminofluorene in liver microsomes by 55–60%. Immune serum and partially purified immune γ-globulin against microsomal NADPH–cytochrome *c* reductase markedly inhibited both the *N*-hydroxylation of 2-acetylaminofluorene and *N*-demethylation of ethylmorphine in liver microsomes from hamsters. These results indicate that *N*-acetylarylamines can be *N*-oxidized by a cytochrome P-450-dependent mixed-function oxidase in liver microsomes.

INTRODUCTION

The well-known toxicities of *N*-acetylarylamines, such as acetanilide and 2-acetylaminofluorene, are believed to result from a toxic metabolite arising through *N*-oxidation (1–3). Whether *N*-oxidation of these substances is mediated by cytochrome P-450 or by a non-cytochrome P-450-dependent flavoprotein is uncertain (4, 5). This paper presents evidence that 2-acetylaminofluorene is *N*-oxidized by a cytochrome P-450 mixed-function oxidase in liver microsomes.

MATERIALS AND METHODS

[9-^{14}C]2-Acetylaminofluorene was obtained from New England Nuclear Corporation with a specific activity of 10.15 mCi/mmole. The purity of the radioactive compound was greater than 99.9% as shown by thin-layer chromatography on silicic acid (solvent system, chloroform–methanol, 97:3). All other reagents were the best available commercial grades. Ouchterlony plates for determination of the double-diffusion patterns of antigen-antibody precipitation reactions were purchased from Hyland Laboratories.

Preparation of microsomal fraction. Microsomes were obtained from male golden Syrian hamsters 6–7 weeks old and male GP mice (22–24 g) from the National Institutes of Health Animal Production Section. Animals were killed by decapitation, and their livers were homogenized with a motor-driven glass-Teflon homogenizer in 2 volumes of 1.15% KCl containing 20 mM Tris-KCl buffer, pH 7.4. The homogenate was centri-

fuged for 20 min at 9000 × *g* in a Sorvall centrifuge, and the supernatant fluid was carefully decanted and recentrifuged for 60 min at 105,000 × *g* in a Spinco model L preparative ultracentrifuge. The microsomal pellet was washed twice by resuspension in Tris-KCl buffer and recentrifuged for 60 min at 105,000 × *g* in a Spinco model L preparative ultracentrifuge. The washed microsomal pellet was resuspended in 0.1 M phosphate buffer, pH 7.4, prior to incubation.

Microsomes were prepared in identical fashion from mice that had been treated with cobaltous chloride (60 mg/kg intraperitoneally daily for 3 days), 24 hr after the last injection. Control mice received injections of 0.9% sodium chloride.

Protein was estimated according to the method of Lowry *et al.* (6).

Preparation of antibody against NADPH–cytochrome c reductase. NADPH–cytochrome *c* reductase was isolated and purified according to the method of Omura and Tabusue (7) from liver microsomes obtained from male rats (Hormone Assay Laboratories, 120–130 g) after administration of phenobarbital (70 mg/kg for 3 days). Female goats were immunized against the 200-fold purified NADPH–cytochrome *c* reductase. Each goat received one intradermal injection of 4 mg of enzyme mixed with Freund's complete adjuvant. Three weeks after the initial injection, an intramuscular injection of 2 mg of the enzyme mixed with Freund's incomplete adjuvant was given and repeated 1 week later. Ten days after the final injection, blood was drawn and serum was obtained by centrifugation of the clotted blood (immune serum). Blood obtained from the goats prior to immunization served as a control in the enzymatic assays (preimmune serum). The γ-globulin fraction of the serum was partially purified by repeated precipitations at 33% saturation with ammonium sulfate, followed by dialysis against 0.02 M phosphate buffer (pH 7.4) to remove the ammonium sulfate (8).

Assay methods. The formaldehyde formed by *N*-demethylation of ethylmorphine was measured by the method of Nash (9).

The incubation mixture, in a total volume of 5.0 ml, contained 20 mM phosphate buffer (pH 7.4), 75 mM KCl, 100 mM NaF, 0.83 mM NADP, 10 mM $MgCl_2$, 20 mM glucose 6-phosphate, 4 enzyme units of glucose 6-phosphate dehydrogenase, and 0.20 mM [9-^{14}C]2-acetylaminofluorene substrate. The sodium fluoride was included to inhibit deacetylation (4). The microsomal protein concentration was 1 mg/ml. Preimmune and immune sera or partially purified γ-globulins were added to the incubation mixture where indicated. Reactions were carried out at 37° in a Dubnoff shaking incubator for 30 min, during which time the reaction rates remained constant.

N - Hydroxy - 2 - acetylaminofluorene was measured by two methods. (a) The ferric chelate of the hydroxamic acid was measured spectrophotometrically according to the method of Booth and Boyland (10). (b) The ^{14}C-labeled hydroxamic acid was purified by thin-layer chromatography (solvent system, chloroform–methanol, 97:3; authentic *N*-hydroxy-2-acetylaminofluorene used as standard) and the radioactivity was measured by scintillation spectrometry (5, 11).

To determine the sensitivity of the reaction to carbon monoxide, the atmosphere in stoppered, iced incubation vessels containing the complete reaction mixture was evacuated five times and replaced with a 90% CO–10% O_2 gas mixture through a two-way stopper. Control vessels were treated similarly with a 90% N_2–10% O_2 gas mixture. The incubation vessels were then incubated as described above.

The amount of cytochrome P-450 in the microsomal fraction was estimated by the method of Omura and Sato (12). The activity of NADPH–cytochrome *c* reductase was measured according to the method of William and Kamin (13), using horse heart cytochrome *c* as the substrate.

RESULTS

The *N*-hydroxylation of [9-^{14}C]2-acetylaminofluorene required oxygen and NADPH and was significantly inhibited by a carbon monoxide atmosphere (90:10) in liver microsomal preparations from the mouse and hamster (Table 1). The metabolite, *N*-hydroxy-[9-^{14}C]2-acetylaminofluorene, was isolated by thin-layer chromatography (5, 11) and measured by scintillation spectrometry. Similar results were obtained by using the

ferric chelation method (10) for measuring the hydroxamic acid.

The structure of the isolated metabolite was verified by mass spectrometry (Fig. 1). The mass spectrum showed a molecular ion (m/e) at 239, and major mass fragments at (M-16), (M-42), (M-58), and (M-74). These mass fragments correspond to the expulsion of oxygen, $COCH_2$, $NHCOCH_3$, and $HONCOCH_3$, respectively, from the parent ion. A further major mass fragment at (M-87) corresponds to the additional loss of the methylene bridge from the fluorene nucleus. Identical mass spectra were obtained from authentic *N*-hydroxy-2-acetylaminofluorene. Authentic 2-acetylaminofluorene gave a mass spectrum with a molecular ion (m/e) at 223 and major mass fragments at (M-42), (M-58), and (M-71), in agreement with the mass spectrum of the isolated metabolite after the expulsion of an oxygen atom (M-16).

TABLE 1

Inhibition by CO of N-hydroxylation of N-2-acetylaminofluorene by mouse and hamster liver microsomes

Experiments were carried out under a 90% CO–10% O_2 atmosphere. The *N*-hydroxy metabolite was isolated by solvent extraction and thin-layer chromatography and measured by scintillation spectrometry. Values are expressed as means ± standard errors of 10 determinations. In control experiments the *C*-oxidation of ethylmorphine by mouse and hamster liver microsomes was inhibited 75% (from 9.2 to 2.3 nmoles/mg/min) and 80% (from 6.5 to 1.3 nmoles/mg/min), respectively, under the same carbon monoxide–oxygen conditions.

Conditions	*N*-Hydroxylation	Inhibition
	nmoles/mg/30 min	%
Mice		
Control	2.90 ± 0.18	
CO-treated	1.74 ± 0.21[a]	40
Hamsters		
Control	4.80 ± 0.34	
CO-treated	1.68 ± 0.12[a]	65

[a] Values significantly different from control ($p < 0.01$).

Liver microsomes are known to hydroxylate 2-acetylaminofluorene in at least three positions of the fluorene nucleus, i.e., positions 3, 5, and 7 (5, 11), apart from the *N*-hydroxylation. A hydroxyl group on position 3, 5, or 7 would give the same molecular ion on mass spectrometry as an *N*-hydroxyl group but would produce a different fragmentation pattern. For example, the expulsion of oxygen (M-16) is characteristic of the presence of an N—O bond in the molecule and does not occur at a hydroxyl group on aromatic ring systems (14), thereby eliminating the possibility of a hydroxyl group on the fluorene nucleus.

Recently Tephly and Hibbeln (15) have

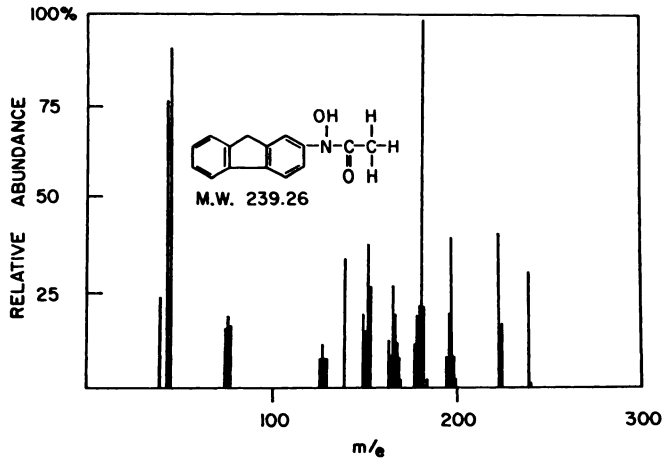

FIG. 1. *Mass spectrum of N-hydroxy-2-acetylaminofluorene*

The *N*-hydroxy metabolite was isolated from the microsomal incubation by solvent extraction and purified by thin-layer chromatography. An LKB-90005 mass spectrometer was used.

shown that prior treatment with cobaltous chloride, an inhibitor of cytochrome P-450 synthesis, markedly reduces the rate of biotransformation of a variety of drugs metabolized by the hepatic cytochrome P-450 mixed-function oxidases. Table 2 demonstrates that similar treatment of mice with cobaltous chloride (60 mg/kg intraperitoneally for 3 days) reduced the hepatic microsomal content of cytochrome P-450 by 55% and inhibited the *N*-hydroxylation of 2-acetylaminofluorene to a comparable extent.

Table 3 shows that immune serum and partially purified immune γ-globulin against NADPH–cytochrome *c* reductase from rat liver microsomes markedly inhibited the *N*-hydroxylation of 2-acetylaminofluorene as well as the *N*-demethylation of ethylmorphine by hepatic microsomes from male hamsters. The antibody preparation was specific for microsome-bound NADPH–cytochrome *c* reductase and showed no cross-reactivity with NADH–cytochrome *c* reductase in rats (Fig. 2). Moreover, only one precipitation band occurred when purified NADPH–cytochrome *c* reductase was allowed to react with the immune serum by the double-diffusion Ouchterlony technique.

DISCUSSION

Whereas *N*-oxidation of secondary and tertiary *N*-alkylarylamines is catalyzed by a flavoprotein-requiring, non-cytochrome P-450, mixed-function oxidase (16), *N*-oxidation of primary arylamines is mediated by a cytochrome P-450 system (17). However, the role of cytochrome P-450 in the oxidation of *N*-acetylarylamines has been unclear (4, 5). Matsushima *et al.* (5) have reported that *N*-hydroxylation of 2-acetylaminofluorene apparently is not a cytochrome P-450-mediated pathway, since a carbon monoxide–air (50:50) atmosphere failed to inhibit formation of the metabolite. Their data are at variance with our present results, which show a marked inhibition of *N*-hydroxylation of 2-acetylaminofluorene by a carbon monoxide–oxygen (90:10) atmosphere (Table 1). This discrepancy may be due to the lower $CO:O_2$ ratio (5:1) used by Matsushima *et al.* The carbon monoxide inhibition of cytochrome P-450 drug-metabolizing enzymes is known to vary greatly, depending on the drug substrate used (18). For example, the aliphatic oxidation of hexobarbital is inhibited about 50%, and the deamination of amphetamine about 90%, under the same

TABLE 2

Decrease in N-hydroxylation of N-2-acetylaminofluorene by liver microsomes from mice treated with cobaltous chloride

Cobaltous chloride was given intraperitoneally in doses of 60 mg/kg for 3 days. Cytochrome P-450 content was calculated using $E = 91\ mM^{-1}\ cm^{-1}$ (12). Values are means ± standard errors of 10 determinations.

Mice	*N*-Hydroxy-2-acetylaminofluorene formed	Cytochrome P-450 content
	nmoles/mg/30 min	*nmoles/mg protein*
Control	3.10 ± 0.29	1.30 ± 0.012
Treated	1.20 ± 0.21[a]	0.58 ± 0.009[a]

[a] Significantly different from control ($p < 0.01$).

TABLE 3

Inhibition of microsomal N-hydroxylation and N-demethylation by immune serum and partially purified γ-globulin against NADPH–cytochrome c reductase

Each incubation contained either 1 ml of preimmune or immune serum per milligram of microsomal protein, or 17 mg of partially purified preimmune or immune γ-globulin per milligram of microsomal protein. Values are means ± standard errors of six determinations.

N-2-Acetylaminofluorene *N*-hydroxylation	*N*-Hydroxy-2-acetylaminofluorene formed	Inhibition
	nmoles/mg/30 min	%
Preimmune serum	7.10 ± 0.35	
Immune serum	1.67 ± 0.12	75
Preimmune γ-globulin	6.60 ± 0.40	
Immune γ-globulin	1.32 ± 0.14	80
Ethylmorphine *N*-demethylation	HCHO formed	Inhibition
	nmoles/mg/10 min	%
Preimmune serum	62.0 ± 4.30	
Immune serum	8.3 ± 0.90	87
Preimmune γ-globulin	58.7 ± 5.20	
Immune γ-globulin	8.1 ± 1.0	86

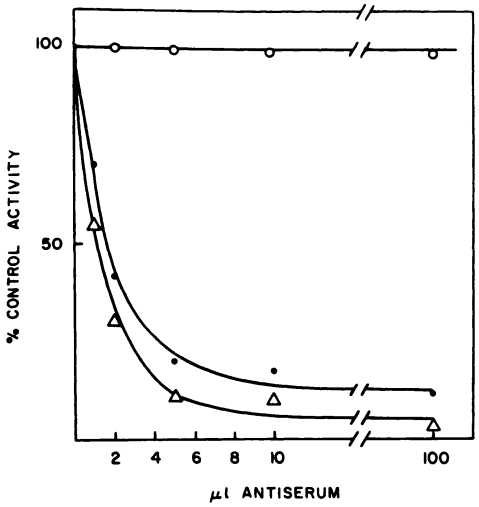

FIG. 2. *Effect of anti-NADPH-cytochrome c reductase immune serum on activities of microsomal NADPH-cytochrome c reductase* (●——●), *purified NADPH-cytochrome c reductase* (△——△), *and microsomal NADH-cytochrome c reductase* (○——○)

The concentrations of microsomal protein and purified NADPH-cytochrome *c* reductase were 0.05 mg and 385 ng, respectively, in a 3.0-ml volume. Control activities were 110 and 24,000 nmoles min^{-1} mg^{-1} for the microsomal and purified NADPH-cytochrome *c* reductase, respectively, and 230 nmoles min^{-1} mg^{-1} for microsomal NADH-cytochrome *c* reductase.

conditions ($CO:O_2$ ratio, 5:1). Since *N*-hydroxylation of 2-acetylaminofluorene appears less sensitive to carbon monoxide than *C*-oxidation of ethylmorphine under the same conditions (Table 1), we consider the lower $CO:O_2$ ratio used by Matsushima *et al.* a possible explanation for the lack of inhibition observed in their study. The lack of significant carbon monoxide inhibition of hydroxylation at C-5 and C-7 of 2-acetylaminofluorene observed by Matsushima *et al.* would support this conclusion.

The use of an inhibitor of cytochrome P-450 synthesis, cobaltous chloride, has proved useful in evaluating the role of cytochrome P-450 in the metabolism of drugs and foreign compounds (15, 19–22). In the present experiments, preliminary treatment of mice with cobaltous chloride significantly decreased the concentration of cytochrome P-450 and concomitantly inhibited *N*-hydroxylation (Table 2), suggesting a dependence of *N*-hydroxylation of 2-acetylaminofluorene on cytochrome P-450.

The importance of NADPH–cytochrome *c* reductase in the cytochrome P-450-dependent electron transport chain in liver microsomes has long been recognized (23–25). Recent studies using immunochemical techniques have further emphasized the fundamental role of NADPH–cytochrome *c* reductase in drug oxidations by the cytochrome P-450 mixed-function oxidases (26–28). For example, immune serum against purified cytochrome *c* reductase inhibits the hydroxylation of aniline, aminopyrine, 3,4-benzpyrene, and ethylmorphine, but not the *N*-oxidation of tertiary alkylarylamines like dimethylaniline (16). These studies have established that immune serum and purified antibody preparations against NADPH–cytochrome *c* reductase can block the microsomal electron transport chain utilizing cytochrome P-450 as the terminal oxidase, thereby preventing drug oxidation. The specificity of the NADPH–cytochrome *c* reductase antibodies for blocking the cytochrome P-450 mixed-function oxidases but not the flavoprotein-mediated amine oxidase was clearly shown by Masters and Ziegler (16) and has been confirmed by us.[1] Thus our present results, demonstrating participation of NADPH–cytochrome *c* reductase in the *N*-oxidation of 2-acetylaminofluorene, strongly support the view that cytochrome P-450 mediates the reaction. This is consistent with the observation that *N*-acetylamines are not substrates for the purified amine oxidase (29) and that the oxidase is not inhibited by carbon monoxide (30).

However, Ziegler (31) recently has shown a protective effect of NADPH–cytochrome *c* reductase in the formation of 2-naphthylhydroxylamine from 2-naphthylamine, a reaction catalyzed by the flavoprotein-mediated amine oxidase (29). The 2-naphthylhydroxylamine undergoes nonenzymatic oxidation to the corresponding nitroso compound, which is then reduced back to the hydroxylamine derivative in the presence of NADPH–cytochrome *c* reductase. However, a similar role of NADPH–cytochrome *c* reductase in

[1] Unpublished observations.

the present studies on the N-oxidation of 2-acetylaminofluorene is not a consideration, because further oxidation of N-hydroxy-2-acetylaminofluorene would result in an obligatory loss of the acetyl group. Thus an NADPH–cytochrome *c* reductase-catalyzed reduction could lead only to a hydroxylamine derivative and could not contribute to the formation of the hydroxamic acid, N-hydroxy-2-acetylaminofluorene.

In summary, the microsome-catalyzed N-oxidation of 2-acetylaminofluorene is inhibited by a carbon monoxide atmosphere, is decreased after treatment of the animals with $CoCl_2$, and is blocked by an antibody against NADPH–cytochrome *c* reductase. The combination of these data leads us to conclude that N-hydroxylation of 2-acetylaminofluorene is catalyzed by a cytochrome P-450-dependent mixed-function oxidase.

Since the toxicity produced by 2-acetylaminofluorene is mediated through an N-hydroxy metabolite (3), it was obviously important to establish the nature of the enzyme pathway leading to the toxic metabolite. The data also explain the inhibition and stimulation of the N-hydroxylation of 2-acetylaminofluorene by piperonyl butoxide (32) and phenobarbital (5), respectively. Piperonyl butoxide is a potent inhibitor and phenobarbital is a well-known inducer of cytochrome P-450 mixed-function oxidases (21, 33).

We recently have observed that the hepatic necrosis produced by another N-acetylarylamine, acetaminophen (*p*-hydroxyacetanilide), is also prevented by prior treatment with piperonyl butoxide and other inhibitors of cytochrome P-450, while the necrosis is potentiated by prior treatment with phenobarbital (19–21). In addition, we have found that the methemoglobinemia and hemolytic anemia produced by acetanilide and its analogues result from the formation of toxic metabolites by cytochrome P-450 oxidases (22). Thus N-oxidation reactions catalyzed by cytochrome P-450 systems may mediate the toxicities of a wide variety of N-acetylarylamines.

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