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Microsomal *N*-Hydroxylation of *p*-Chloroacetanilide

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SUMMARY

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Microsomes obtained from hamster liver catalyze the *N*-hydroxylation of *p*-chloroacetanilide. The reaction requires NADPH and is inhibited by a carbon monoxide–oxygen atmosphere, indicating that it is catalyzed by a cytochrome P-450-dependent mixed-function oxidase. The enzyme activity in hamster microsomes is induced by prior treatment of the animals with 3-methylcholanthrene but not with phenobarbital, and is inhibited by piperonyl butoxide. The yield of *N*-hydroxy metabolite is enhanced by the presence of sodium fluoride in the reaction mixture; this effect appears to be due to a selective stimulation of *N*-hydroxylation and not to an inhibition of deacetylation of *N*-hydroxy-*p*-chloroacetanilide. These results demonstrate that the microsomal enzyme system catalyzing the *N*-hydroxylation of *p*-chloroacetanilide and the formation of the arylating, hepatotoxic metabolite of acetaminophen share many distinctive characteristics. The close correlation between these systems supports the hypothesis that the hepatotoxic metabolite of acetaminophen results from initial *N*-hydroxylation followed by immediate loss of water to yield the chemically reactive acetimidoquinone.

INTRODUCTION

The toxicities of several polycyclic *N*-acetylarylamines are believed to result from reactive metabolites formed through *N*-hydroxylation (1-3). For example, 2-acetylaminofluorene has been shown in numerous studies to be metabolized to *N*-hydroxy-2-acetylaminofluorene (1-5), which after sulfate conjugation is a potent arylating agent (6). The amounts of fluorene derivatives bound to hepatic protein and nucleic acids of animals receiving 2-acetylaminofluorene or *N*-hydroxy-2-acetylaminofluorene have generally paralleled susceptibility to hepatic carcinogenesis (6-8). The *N*-hydroxylation of 2-acetylaminofluorene is catalyzed by a cytochrome P-450-dependent mixed-function oxidase located in hepatic microsomes (1-3, 9).

Recent studies have indicated that the fulminant hepatic necrosis caused by acetaminophen (*p*-hydroxyacetanilide) results from the formation of a toxic arylating metabolite (10-15). We have postulated that the hepatotoxic metabolite is the chemically reactive acetimidoquinone derivative from *N*-hydroxyacetaminophen (16). However, the role of *N*-hydroxylation in the metabolism of monocyclic *N*-acetylarylamines has been questioned (17-19). Recently we found that the *N*-hydroxy analogue of *p*-chloroacetanilide, in contrast to *N*-hydroxyacetaminophen, is chemically stable and can be isolated and assayed. We have taken advantage of this fact to demonstrate conclusively that mon-

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ocyclic *N*-acetylarylamines such as *p*-chloroacetanilide are *N*-hydroxylated by hepatic cytochrome P-450 oxidases.

MATERIALS AND METHODS

[1-^{14}C]Acetyl chloride was obtained from New England Nuclear Corporation (specific activity, 30.2 mCi/mmole). Pyridine nucleotides and glucose 6-phosphate dehydrogenase were obtained from Boehringer/Mannheim Corporation; glucose 6-phosphate was supplied by Sigma Chemical Company; and thin-layer chromatographic plates were purchased from Analtech. All other chemicals were of the purest grade commercially available.

Synthesis of N-hydroxy-p-chloroacetanilide. *N*-Hydroxy-*p*-chloroacetanilide was synthesized from *p*-chloronitrobenzene in two steps. *p*-Chloronitrobenzene was reduced by zinc dust in the presence of ammonium chloride to yield *p*-chlorophenylhydroxylamine, which was isolated and recrystallized (20). The *p*-chlorophenylhydroxylamine was then dissolved in ether and selectively *N*-acetylated by treatment with acetyl chloride in the presence of a slurry of sodium bicarbonate as described by Priyadarshini and Tandon (21). *N*-Hydroxy-*p*-chloroacetanilide was purified by crystallization from ether–benzene, and its identity was confirmed as described in RESULTS. For the synthesis of *N*-hydroxy-*p*-chloroacetanilide labeled with ^{14}C in the acetyl group, [^{14}C]carbonyl-labeled acetyl chloride was used instead of the unlabeled reagent. The ^{14}C-labeled *N*-hydroxy-*p*-chloroacetanilide was purified by preparative thin-layer chromatography on silica gel (solvent system, chloroform–methanol, 95:5). The radiochemical purity of the isolated material was greater than 99.5% as shown by analytical thin-layer chromatography on silica gel GF [solvent systems, chloroform–methanol, 95:5, and chloroform–methanol–ammonium hydroxide (15 M), 92:7:0.5].

Synthesis of [^{14}*C*]*p-chloroacetanilide.* *p*-Chloro[*carbonyl*-^{14}C]acetanilide was synthesized by allowing [1-^{14}C]acetyl chloride to react with an excess of *p*-chloroaniline in ether (22). The resulting [^{14}C]*p*-chloroacetanilide was purified by washing the ether phase (50 ml) five times with an equal volume of 0.1 M hydrochloric acid, 5 times with an equal volume of 0.1 M NaOH, and twice with 2 volumes of distilled water. The purity of the radiolabeled compound obtained after evaporation of the ether was greater than 99% as shown by analytical thin-layer chromatography on silica gel GF (solvent system, chloroform–methanol, 95:5).

Preparation of microsomal fraction. Male golden Syrian hamsters (60 g) were obtained from the National Institutes of Health Animal Production Section and maintained on Purina laboratory chow and water ad libitum. Animals were killed by decapitation, and their livers were homogenized at 4° with a motor-driven glass-Teflon homogenizer in 6 volumes of 1.15% KCl containing 20 mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged 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 by resuspension in Tris-KCl buffer and recentrifugation at 105,000 × *g* for 40 min. The washed microsomal pellet was resuspended in 0.05 M phosphate buffer, pH 7.4, prior to incubation. Microsomes were also prepared from hamsters that had been treated with 3-methylcholanthrene (20 mg/kg intraperitoneally, in corn oil, daily for 3 days), or phenobarbital (80 mg/kg intraperitoneally, daily for 3 days). The animals were killed 24 hr after the last injection. Other hamsters received piperonyl butoxide (1500 mg/kg intraperitoneally) 30 min prior to death.

Assay methods. The incubation mixture contained 6 mg of hepatic microsomes, 150 μmoles of potassium phosphate (pH 7.4), 190 μmoles of potassium chloride, 2 μmoles of NADP, 3 μmoles of magnesium chloride, 9 μmoles of glucose 6-phosphate, 4 units of glucose 6-phosphate dehydrogenase, and 1.5 μmoles of [^{14}C]*p*-chloroacetanilide (6 mCi/mmole) in a total volume of 3 ml. Sodium fluoride (300 μmoles) was routinely included in the incubation mixture, since this compound has been shown to increase the yield of *N*-hydroxy metabo-

lites of polycyclic *N*-acetylarylamines (17). Reactions were carried out at 37° in a Dubnoff shaking incubator. The reaction was terminated by rapid cooling in an ice bath.

In the standard assay for [^{14}C]*N*-hydroxy-*p*-chloroacetanilide, nonradioactive *N*-hydroxy-*p*-chloroacetanilide (250 μg) was added to each flask after incubation to minimize adsorption losses in the subsequent purification procedure. Metabolites and unreacted [^{14}C]*p*-chloroacetanilide were obtained by extracting the incubation mixture with 10 ml of ether three times. The combined ether phases were evaporated under nitrogen, and the residue was dissolved in 20 ml of 0.75 M ammonium hydroxide. Unreacted [^{14}C]*p*-chloroacetanilide was removed by washing the ammonium hydroxide phase twice with 10 ml of ether. The ether phases were discarded. The ammonium hydroxide phase was neutralized with 10 ml of 2 M potassium dihydrogen phosphate, and [^{14}C]*N*-hydroxy-*p*-chloroacetanilide was extracted by shaking the aqueous phase three times with 10 ml of ether. The combined ether phases were evaporated under nitrogen, and the residue was spotted on a 5 × 20 cm, 250-μm-thick silica gel GF plate. The silica gel plate was developed using an ether–heptane (75:25) solvent system. The region corresponding to *N*-hydroxy-*p*-chloroacetanilide was scraped from the plate, and the radioactivity in this spot was measured by scintillation spectrometry.

To determine the specific activity of [^{14}C]*N*-hydroxy-*p*-chloroacetanilide, the radioactivity in the sample was measured by liquid scintillation spectroscopy. *N*-Hydroxy-*p*-chloroacetanilide was measured by addition of 5 ml of a methanolic solution of ferric chloride (0.2%) and determination of the optical density of the ferric chelate at 530 nm. The amount of *N*-hydroxy-*p*-chloroacetanilide in the sample was calculated from the millimolar extinction coefficient (1.44 mM^{-1} cm^{-1}) of authentic *N*-hydroxy-*p*-chloroacetanilide determined under the same conditions. Protein was estimated according to Lowry *et al.* (23), using serum albumin as a standard. Kinetic calculations were performed on a Wang 600 calculator, using a linear regression analysis program.

RESULTS

Identification of N-hydroxy-p-chloroacetanilide. The structure of synthetic *N*-hydroxy-*p*-chloroacetanilide was verified by electron impact mass spectrometry, chemical ionization mass spectrometry, and nuclear magnetic resonance spectrometry. The electron impact mass spectrum showed 2 molecular ions (*m/e* at 185 and 187 with peak heights in the ratio of about 3:1) corresponding to the isotopes of chlorine. Major mass fragments were found at (*M* − 16), (*M* − 42), and (*M* − 58), which correspond to the loss of oxygen, $COCH_2$, and oxygen plus $COCH_2$, respectively. Although the phenolic metabolites of *p*-chloroacetanilide may give the same parent ions as the *N*-hydroxy derivative, expulsion of oxygen (*M* − 16) from the mass fragments is characteristic of a N—O bond in the molecule and does not occur with phenolic groups (24). Similar fragmentation patterns have been noted for *N*-hydroxy-2-acetylaminofluorene (9). Chemical ionization mass spectrometry of the synthetic *N*-hydroxy-*p*-chloroacetanilide showed molecular ions at 186 and 188, corresponding to the *M* + 1 peaks. Major mass fragments were found at *m/e* 168 and 170, corresponding to the loss of water.

Further confirmation that the synthesized compound was the *N*-hydroxy derivative was obtained with nuclear magnetic resonance spectrometry. The spectrum showed a singlet at 2.45 ppm, which contained 3 protons, and two doublets at 7.52 and 7.80 ppm, each of which contained 2 protons. The singlet represents the 3 protons of the acetyl group, and the two doublets indicate the presence of 4 protons attached to the aromatic ring.

Preliminary studies on the metabolism of [^{14}C]*p*-chloroacetanilide in the hamster liver microsomes revealed a metabolite whose R_F was identical with that of authentic *N*-hydroxy-*p*-chloroacetanilide when chromatographed on silica gel in the four solvent systems listed in Table 1. To verify the occurrence of *N*-hydroxy-*p*-chloroacetanilide as a metabolite of *p*-

TABLE 1

Constant specific activity of proposed [^{14}C]N-hydroxy-p-chloroacetanilide metabolite during sequential thin-layer chromatography

[^{14}C]p-Chloroacetanilide (1.5 μmoles) was incubated with liver microsomes (2.0 mg per ml) from 3-methylcholanthrene-treated hamsters in the presence of sodium fluoride (300 μmoles), phosphate buffer (150 μmoles, pH 7.4), and an NADPH-generating system. After a 20-min incubation, nonradioactive *N*-hydroxy-*p*-chloroacetanilide (250 μg) was added. [^{14}C]*N*-Hydroxy-*p*-chlororacetanilide was extracted as described in MATERIALS AND METHODS and sequentially subjected to thin-layer chromatography on silica gel using four different solvent systems. The specific activity of the [^{14}C]*N*-hydroxy-*p*-chloroacetanilide was determined after each thin-layer chromatographic procedure as described in MATERIALS AND METHODS.

Solvent system	R_f of *N*-hydroxy-*p*-chloro-acetanilide	Specific activity of metabolite
		μCi/μmole
1. Ether–heptane (75:25)	0.38	0.066
2. Chloroform–methanol (95:5)	0.53	0.062
3. Chloroform–acetone–NH_4OH (15 M) (50:50:1)	0.67	0.064
4. Chloroform–methanol–NH_4OH (15 M) (92:7:0.5)	0.59	0.068

chloroacetanilide, hamster liver microsomes (1 g) were incubated with [^{14}C]p-chloroacetanilide (250 μmoles) in the presence of an NADPH-generating system and potassium phosphate buffer (0.05 M, pH 7.4, 500 ml). The NADPH-generating system was omitted from the control incubation. The metabolite, which was absent from the control incubation, was isolated as described in MATERIALS AND METHODS except that nonradioactive *N*-hydroxy-*p*-chloroacetanilide was not added to the incubation mixture. The electron impact mass spectrum of this metabolite (Fig. 1) was identical with that of the authentic *N*-hydroxy-*p*-chloroacetanilide.

Estimation of microsomal N-hydroxylation of p-chloroacetanilide. The validity of the standard assay for [^{14}C]*N*-hydroxy-*p*-chloroacetanilide described in MATERIALS AND METHODS was confirmed by an isotope dilution experiment (Table 1). The [$^{+}$C]*N*-hydroxy-*p*-chloroacetanilide metabolite was isolated and purified by the standard assay system after the addition of nonradioactive *N*-hydroxy-*p*-chloroacetanilide. The specific activity was 0.066 μCi/μmole (Table 1). Sequential rechromatography of this material in three additional solvent systems (Table 1) did not significantly alter the specific activity. Thus the isolation procedure used in the standard assay yields radiochemically pure metabolite. In other experiments [^{14}C]*N*-hydroxy-*p*-chloroacetanilide was added to microsomes at 0° in the approximate amounts produced in the assay mixture and estimated by the standard assay. Recovery of [^{14}C]*N*-hydroxy-*p*-chloroacetanilide in five experiments was 70 ± 3% because of the partitioning of *N*-hydroxy-*p*-chloroacetanilide between ammonium hydroxide and ether. All calculations for the production or disappearance of *N*-hydroxy-*p*-chloroacetanilide have been corrected for this loss.

The rate of formation of [^{14}C]*N*-hydroxy-*p*-chloroacetanilide in the standard incubation system was shown to be constant for 10 min and proportional to microsomal protein concentration up to 2 mg/ml. The possibility that the metabolite was converted to other metabolites during the incubation was evaluated by measuring the disappearance of [^{14}C]*N*-hydroxy-*p*-chloroacetanilide (Fig. 2). In the absence of *p*-chloroacetanilide [^{14}C]*N*-hydroxy-*p*-chloroacetanilide was metabolized in the incubation mixture. Only about 64% of the quantity initially present could be recovered after a 10-min incubation. In the presence of *p*-chloroacetanilide, however, *N*-hydroxy-*p*-chloroacetanilide was not metabolized; over 90% of the quantity initially present could be recovered after a 10-min incubation.

Effect of incubation conditions and animal treatments on N-hydroxylation of p-chloroacetanilide by hamster microsomes. Table 2 shows that the *N*-hydroxylation of *p*-chloroacetanilide by hamster liver microsomes was abolished by heating the microsomes to 80° for 5 min and markedly inhibited by the omission of NADPH or oxygen from the incubation mixture.

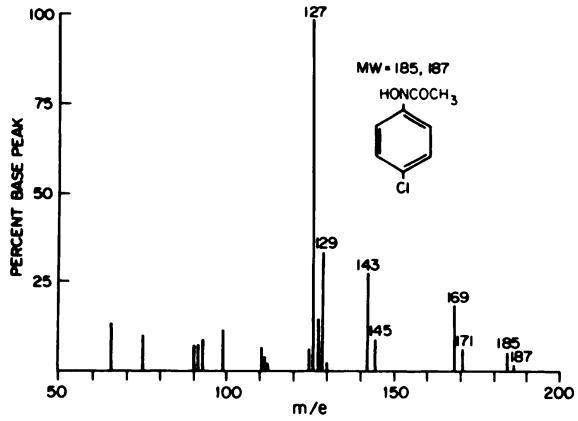

FIG. 1. *Electron impact mass spectrum of N-hydroxy-p-chloroacetanilide metabolite*

The *N*-hydroxy-*p*-chloroacetanilide metabolite was isolated from the microsomal incubation by solvent extraction and purified by thin-layer chromatography. An LKB 9000 mass spectrometer was used.

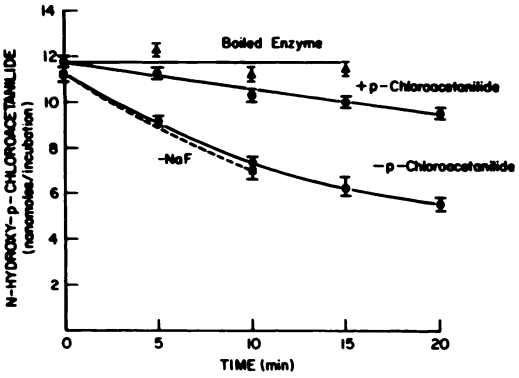

FIG. 2. *Disappearance of [^{14}C]N-hydroxy-p-chloroacetanilide with time from standard incubation mixtures.*

[^{14}C]*N*-Hydroxy-*p*-chloroacetanilide was added to a standard incubation mixture containing microsomes and an NADPH-generating system, with and without 0.5 mM *p*-chloroacetanilide. At various time intervals [^{14}C]*N*-hydroxy-*p*-chloroacetanilide was determined. The effect of omission of sodium fluoride from the incubation mixture which did not contain *p*-chloroacetanilide is also shown (■- - -■). The control incubations contained microsomes which had been heated to 90° for 10 min and *p*-chloroacetanilide (▲——▲). Each point is the average of two determinations.

NADH was not as effective as NADPH. The reaction was also markedly inhibited by a carbon monoxide–oxygen atmosphere. These data suggest that the microsomal *N*-hydroxylation of *p*-chloroacetanilide, like that of 2-acetylaminofluorene (9), is catalyzed by a cytochrome P-450-dependent mixed-function oxidase.

Sodium fluoride is known to increase the yield of *N*-hydroxy metabolites of polycyclic *N*-acetylarylamines when added to microsomal incubations (17, 25). This effect has been attributed to inhibition of microsome-catalyzed deacetylation (17). Omission of sodium fluoride from the incubation mixture for the *N*-hydroxylation of *p*-chloroacetanilide was found to decrease significantly the yield of *N*-hydroxy-*p*-chloroacetanilide (Table 2). However, as shown in Fig. 2, the absence of sodium fluoride from the incubation mixture did not increase the rate of disappearance of *N*-hydroxy-*p*-chloroacetanilide. The effect of sodium fluoride on the yield of *N*-hydroxy-*p*-chloroacetanilide in the microsome-catalyzed reaction thus appears to be associated with the formation of the metabolite and not with its further metabolism. One could postulate that *p*-chloroacetanilide is normally deacetylated to *p*-chloroaniline, which might inhibit hydroxamic acid formation, and that sodium fluoride increases the yield of hydroxamic acid by inhibiting the deacetylation of *p*-chloroacetanilide to *p*-chloroaniline. This seems unlikely, however, because the formation of the hydroxamic acid is not increased by addition to the incubation of bis(*p*-nitrophenyl) phosphate, a known inhibitor of the deacetylation of *p*-chloro-

TABLE 2

Effect of incubation conditions on N-hydroxylation of p-chloroacetanilide by hamster liver microsomes

The complete incubation mixture (control) contained liver microsomes from 3-methylcholanthrene-treated hamsters (2 mg/ml), NADP (2 μmoles), an NADPH-generating system, [^{14}C]p-chloroacetanilide (1.5 μmoles, 1.4 μCi/μmole), NaF (300 μmoles), and an air atmosphere. In the $-O_2$ and $+CO-O_2$ experiments the air atmosphere was replaced by repeated evacuation and flushing with N_2 or with a 90% CO-10% O_2 atmosphere, respectively. In parallel experiments the $CO-O_2$ atmosphere inhibited the *C*-oxidation of ethylmorphine by 80% (from 6.0 to 1.2 nmoles/mg/min). In the heat-treated microsome experiments the microsomes were first incubated at 80° for 5 min. The values given are the mean ± the range of two determinations.

Conditions	*N*-Hydroxylation	Inhibition
	pmoles/mg/10 min	%
Control	325 ± 22	
−NADPH	3 ± 1	99
$-O_2$ (100% N_2)	26 ± 4	81
$+N_2-O_2$ (9:1)	313 ± 27	0
$+CO-O_2$ (9:1)	135 ± 2	58
−NADPH + NADH	71 ± 2	78
−NaF	159 ± 16	51
Heat-treated microsomes	4 ± 2	99

acetanilide (26). This interpretation is also supported by the observation that the *N*-hydroxylation of *p*-chloroacetanilide was constant for at least 10 min in both the presence and absence of sodium fluoride. In parallel experiments sodium fluoride (100 mM) did not significantly affect the hamster liver microsome-catalyzed *N*-demethylation of ethylmorphine, the *O*-demethylation of *p*-nitroanisole, or the *C*-oxidation of *p*-chloroacetanilide [formation of *m*-hydroxy-*p*-chloroacetanilide (data not shown)].

The *N*-hydroxylation reaction by liver microsomes from normal hamsters had a K_m value of 22 μM and a V_{max} of 94 pmoles/mg/10 min (Fig. 3). Prior treatment of the animals with phenobarbital did not significantly alter either of these kinetic parameters (V_{max}, 88 pmoles/mg/10 min; K_m, 17 μM). In contrast, treatment with 3-methylcholanthrene markedly increased the V_{max}, by 3.5-fold, and apparently decreased the K_m value slightly, to 9 μM. Piperonyl butoxide treatment of the hamsters resulted in decreased *N*-hydroxylation (V_{max}, 43 pmoles/mg/10 min) and an increased K_m (35 μM).

DISCUSSION

The role of *N*-hydroxylation in the metabolism of monocyclic *N*-acetylarylamines has been unclear. Booth and Boyland (17) examined the capacity of rabbit liver microsomes to *N*-hydroxylate a variety of *N*-acetylarylamines, including acetanilide. Under conditions which gave maximal yields of 4-(*N*-hydroxyacetamido)biphenyl from 4-acetamidobiphenyl, the conversion of acetanilide to *N*-hydroxyacetanilide could not be detected. Moreover, Poirier *et al.* (18) administered acetanilide and its *p*-vinyl, *p*-fluoro, and *p*-ethoxy derivatives to dogs and reported that no *N*-hydroxy amides could be detected in the urine. Similarly, Kiese and Lenk (19) reported a lack of the *N*-hydroxy metabolite of *p*-chloracetanilide in the urine of rabbits fed this compound. On the other hand, Troll and Belman (27, 28) administered acetanilide and phenacetin to humans and reported the occurrence of the *N*-hydroxy derivative as urinary metabolites. However, the results of Troll and Belman have been questioned (3) because their method is not specific for *N*-hydroxy derivatives.

The present data clearly indicate that monocyclic *N*-acetylarylamines such as *p*-chloroacetanilide are metabolized to their *N*-hydroxy derivatives by hamster microsomes. The reaction requires NADPH and oxygen and is inhibited by carbon monoxide, indicating that it is catalyzed by a cytochrome P-450-dependent mixed-function oxidase. The enzyme activity is induced in hamsters by 3-methylcholanthrene but not by phenobarbital, and is inhibited by piperonyl butoxide. The presence of high concentrations of sodium fluoride (0.1 M) in the reaction mixture markedly enhanced the yield of *N*-hydroxy-*p*-chloroacetanilide. Although it has been suggested that sodium fluoride enhances the *N*-hydroxylation of polycyclic

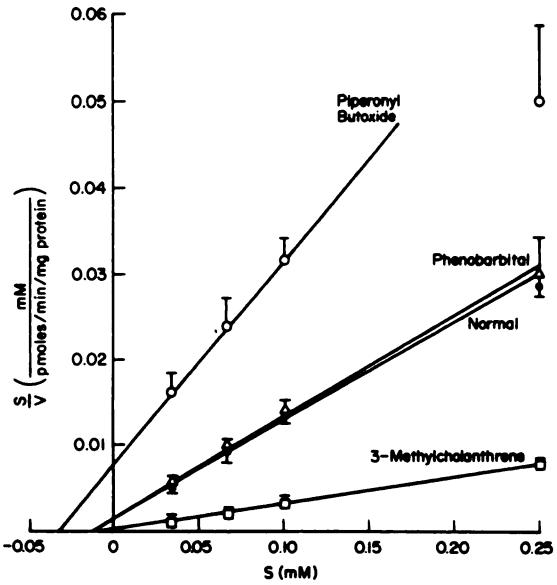

FIG. 3. *Effects of treatment of hamsters with 3-methylcholanthrene, phenobarbital, and piperonyl butoxide on N-hydroxylation of [^{14}C]p-chloroacetanilide.*

Animals were treated with either 3-methylcholanthrene, phenobarbital, or piperonyl butoxide as described in MATERIALS AND METHODS. Incubation conditions were the same as described in MATERIALS AND METHODS except that the concentration of *p*-chloroacetanilide was varied as indicated. Each point is the mean value of four experiments using microsomes from different animals.

N-acetylarylamines by inhibiting the deacetylation of the *N*-hydroxy derivatives (17, 25), our studies show that sodium fluoride does not affect the disappearance of *N*-hydroxy-*p*-chloroacetanilide from the the incubation mixture. Thus the enhancement of *N*-hydroxylation of monocyclic *N*-acetylarylamines is due to a stimulatory effect on mixed-function oxidase activity. Nevertheless, sodium fluoride does not alter the oxidative demethylation of ethylmorphine and *p*-nitroanisole, nor does it affect the conversion of *p*-chloroacetanilide to its phenolic metabolites. The effect of sodium fluoride therefore may be selective for *N*-hydroxylation reactions.

We have previously suggested that *N*-hydroxylation of acetaminophen immediately leads to loss of water and formation of the chemically reactive acetimidoquinone, which mediates the fulminant hepatic necrosis caused by large doses of acetaminophen in man and experimental animals (see ref. 16 for review). Liver necrosis is associated with the formation of a toxic arylating metabolite of acetaminophen (10–14) that combines covalently with hepatic glutathione and, after the glutathione has been depleted from the liver, with hepatic macromolecules (13–15). The formation of the hepatotoxic metabolite of acetaminophen is catalyzed by a microsomal cytochrome P-450-dependent mixed-function oxidase (12, 14, 15), which in hamsters is induced by 3-methylcholanthrene but not by phenobarbital, and is inhibited by piperonyl butoxide (14, 15).

The present studies demonstrate that the microsomal enzyme system catalyzing the *N*-hydroxylation of *p*-chloroacetanilide and the formation of the arylating, hepatotoxic metabolite of acetaminophen share several distinctive characteristics. Both enzyme systems are microsomal cytochrome P-450-dependent mixed-function oxidases, the activities of which are enhanced about 2-fold by inclusion of sodium fluoride in the incubation mixture. Both enzyme activities are induced in hamster liver by 3-methylcholanthrene but not by phenobarbital. Both are inhibited by piperonyl butoxide. The close correlation between these

systems further supports the hypothesis that the hepatotoxic metabolite of acetaminophen results from initial *N*-hydroxylation followed by immediate loss of water to yield the reactive acetimidoquinone.

REFERENCES

1. Miller, J. A. (1970) *Cancer Res.*, **30**, 559-576.
2. Miller, J. A. & Miller, E. C. (1969) *Prog. Exp. Tumor Res.*, **11**, 273-301.
3. Weisburger, J. H. & Weisburger, E. K. (1973) *Pharmacol. Rev.*, **25**, 1-66.
4. Cramer, J. W., Miller, J. A. & Miller, E. C. (1960) *J. Biol. Chem.*, **235**, 885-888.
5. Irving, C. C. (1962) *Biochim. Biophys. Acta*, **675**, 564-566.
6. DeBaun, J. R., Smith, Y. R., Miller, E. C. & Miller, J. A. (1970) *Science*, **167**, 184-186.
7. Marroquin, F. & Farber, E. (1965) *Cancer Res.*, **27**, 1262-1269.
8. Irving, C. C., Veazey, R. A. & Williard, R. F., (1967)*Cancer Res.*, **27**, 720-725.
9. Thorgeirsson, S. S., Jollow, D. J., Sasame, H. A., Green, I. & Mitchell, J. R. (1973) *Mol. Pharmacol.*, **9**, 398-404.
10. Mitchell, J. R., Jollow, D. J., Davis, D. C., Gillette, J. R. & Brodie, B. B. (1973) *J. Pharmacol. Exp. Ther.*, **187**, 185-194.
11. Jollow, D. J., Mitchell, J. R., Potter, W. Z., Davis, D. C., Gillette, J. R. & Brodie, B. B. (1973) *J. Pharmacol. Exp. Ther.*, **187**, 195-202.
12. Potter, W. Z., Davis, D. C., Mitchell, J. R., Jollow, D. J., Gillette, J. R. & Brodie, B. B. (1973) *J. Pharmacol. Exp. Ther.*, **187**, 203-210.
13. Mitchell, J. R., Jollow, D. J., Potter, W. Z., Gillette, J. R. & Brodie, B. B. (1973) *J. Pharmacol. Exp. Ther.*, **187**, 211-217.
14. Potter, W. Z., Thorgeirsson, S. S., Jollow, D. J. & Mitchell, J. R. (1974) *Pharmacology*, **12**, 129-143.
15. Jollow, D. J., Thorgeirsson, S. S., Potter, W. Z., Hashimoto, M. & Mitchell, J. R. (1974) *Pharmacology*, **12**,, 251-271.
16. Mitchell, J. R. & Jollow, D. J. (1975) *Gastroenterology*, **68**, 392-410.
17. Booth, J. & Boyland, E. (1964) *Biochem. J.*, **91**, 362-369.
18. Poirier, L. A., Miller, J. A. & Miller, E. C. (1963) *Cancer Res.*, **23**, 790-800.
19. Kiese, M. & Lenk, W. (1971) *Biochem. Pharmacol.*, **20**, 379-391.
20. Kamm, O. (1941) in *Organic Synthesis* (Gilman, H. & Blatt, A., eds.), Vol. 1, pp. 445-447, Wiley, New York.
21. Priyadarshini, U. & Tandon, S. G. (1967) *J. Chem. Engng Data*, **12**, 143-144.
22. Wallach, O. (1882) *Justus Liebigs Ann. Chem.*, **214**, 234-256.
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
24. McLafferty, F. W. (1967) in *Interpretation of Mass Spectra*, p. 216-217, WA Benjamin Co., New York.
25. Irving, C. C. (1964) *J. Biol. Chem.*, **239**, 1589-1596.
26. Di Chiara, G., Hinson, J. A., Potter, W. Z., Jollow, D. J. & Mitchell, J. R. (1973) *Fed. Proc.*, **32**, 305.
27. Troll, W. & Belman, S. (1967) in *Bladder Cancer, a Symposium* (K. F. Lampe, ed.), pp. 35-44, Aesculapus, Birmingham.
28. Belman, S., Troll, W., Teebor, G. & Mukai, F. (1968) *Cancer Res.*, **28**, 535-542.