MOLECULAR PHARMACOLOGY, 13, 625–633

Studies on the Microsomal Formation of Arylating Metabolites of Acetaminophen and Phenacetin

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(Received October 22, 1976)
(Accepted February 22, 1977)

SUMMARY

HINSON, JACK A., NELSON, SIDNEY D. & MITCHELL, JERRY R. (1977) Studies on the microsomal formation of arylating metabolites of acetaminophen and phenacetin. *Mol. Pharmacol.*, 13, 625–633.

A chemically reactive metabolite of phenacetin is formed by a cytochrome P-450 in hamster liver microsomes by a mechanism that is different from the generation of the chemically reactive metabolite from acetaminophen. The V_{max} of covalent binding of phenacetin exceeds that of acetaminophen, showing that phenacetin is not first deethylated to acetaminophen, which is then activated. Previous treatment with 3-methylcholanthrene increases the V_{max} of covalent binding for acetaminophen but decreases the V_{max} of covalent binding for phenacetin, even though it increases the V_{max} of deethylation of phenacetin to acetaminophen. Previous treatment with phenobarbital increases the V_{max} of covalent binding for phenacetin without increasing the same parameter for acetaminophen. Addition of sodium fluoride (0.1 M) increases the rate of covalent binding for acetaminophen but decreases it for phenacetin. When covalent binding is prevented by trapping the reactive metabolites with glutathione during incubations carried out under $^{18}O_2$ atmospheres, reductive cleavage by Raney nickel of the glutathione conjugates formed from either phenacetin or acetaminophen yields acetaminophen; however, the acetaminophen from the phenacetin-glutathione incubations contains about 50% ^{18}O in position 4, whereas the acetaminophen from the acetaminophen-glutathione incubations contains virtually no ^{18}O. These findings are consistent with the hypothesis that acetaminophen is converted to *N*-hydroxyacetaminophen, which dehydrates to yield the arylating species, acetimidoquinone, whereas the reactive species from phenacetin arises by epoxidation.

INTRODUCTION

Recent studies have shown that the hepatic necrosis caused by acetaminophen (4-hydroxyacetanilide) results from the formation of a toxic arylating metabolite (1–5). Our laboratory has postulated that the initial event in the production of this hepatotoxic metabolite is *N*-hydroxylation of the parent drug to *N*-hydroxyacetaminophen, which spontaneously dehydrates to yield acetimidoquinone (6), a known arylating agent (7). In support of this view, the microsomal formation of the arylating metabolite of acetaminophen is similar to the microsomal *N*-hydroxylation of *p*-chloroacetanilide and phenacetin, stable analogues of the proposed *N*-hydroxymetabolite of acetaminophen which do not spontaneously rearrange to acetimidoquinone at pH 7.4 (8, 9). Like the formation of the toxic metabolite of acetaminophen, the *N*-hydroxylation of *p*-chloroacetanilide and phenacetin *in vitro* was induced by treat-

ISSN 0026-895X

ment of hamsters with 3-methylcholanthrene and inhibited by treatment with piperonyl butoxide. Treatment of hamsters with phenobarbital, however, had little effect on the kinetics of covalent binding of acetaminophen or on the *N*-hydroxylation of *p*-chloroacetanilide and phenacetin (5, 8, 9). Also, sodium fluoride (0.1 M) markedly stimulated the *N*-hydroxylation of *p*-chloroacetanilide and phenacetin as well as the covalent binding of acetaminophen (3, 8, 9).

Phenacetin (4-ethoxyacetanilide) as well as acetaminophen causes centrilobular hepatic necrosis in experimental animals (1, 6). Moreover, phenacetin has been implicated in the pathogenesis of the interstitial nephritis and renal pelvic tumors seen in abusers of this drug (10–12). The mechanisms of these toxicities are unknown but might be related to the formation of arylating metabolites from phenacetin (6, 13).

The present report demonstrates the formation of an arylating metabolite from phenacetin by liver microsomes from hamsters, the species most susceptible to phenacetin and acetaminophen liver injury (1, 6). It also compares the reaction mechanisms by which the arylating metabolites of phenacetin and acetaminophen are formed and shows that the two metabolites are generated by different mechanisms.

MATERIALS AND METHODS

Phenacetin (generally ^{3}H-labeled; specific activity, 86 mCi/mmole; 99% pure) and acetaminophen (generally ^{3}H-labeled; specific activity, 99 mCi/mmole; 99% pure) were obtained from New England Nuclear. ^{14}C-Acetyl-labeled *N*-hydroxyphenacetin was synthesized as previously described (9). $^{18}O_2$ gas (99% excess) was a product of Bio-Rad Laboratories. Glutathione, NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Company. Thin-layer chromatographic plates were obtained from Analtech. All other chemicals were of the purest grade commercially available.

Preparation of microsomal fraction. Male golden Syrian hamsters (60 g) were obtained from the NIH Animal Production Section and maintained on Purina laboratory chow and water *ad libitum*. Animals were killed by decapitation, and their livers were homogenized in 6 volumes of 1.15% KCl containing 20 mM Tris-HCl buffer, pH 7.4, at 4°, with a motor-driven glass-Teflon homogenizer. 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 recentrifuged at 105,000 × *g* for 40 min. The washed microsomal pellet was resuspended in 0.05 M sodium phosphate buffer, pH 7.4, prior to incubation. Hepatic 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 of 3-methylcholanthrene or phenobarbital. Other animals received piperonyl butoxide (1500 mg/kg intraperitoneally, 30 min prior to death). In comparing the effects of these treatments, animals from the same group were used.

Assay methods. The incubation mixtures (3 ml) contained 6 mg of hepatic microsomal protein, 150 μmoles of sodium phosphate (pH 7.4), 1 μmole of NADP, 15 μmoles of magnesium chloride, 31 μmoles of glucose 6-phosphate, 2 units of glucose 6-phosphate dehydrogenase, and 3 μmoles of either [^{3}H]phenacetin (500 cpm/nmole) or [^{3}H]acetaminophen (500 cpm/nmole). Duplicate mixtures were incubated for 10 min at 37° in a Dubnoff shaking incubator. The reaction was terminated by rapidly cooling the mixtures in an ice bath, followed by addition of 4 ml of methanol to precipitate the protein. After addition of methanol the reaction mixtures were poured into a 13-ml centrifuge tube and centrifuged for 15 min at 1000 × *g*. The supernatant was discarded, and the protein was washed repeatedly with methanol until no further radioactivity could be removed (2, 3). The extracted protein was then dissolved in 1 M NaOH, the radioactivity of an aliquot was

determined by liquid scintillation spectrometry, and the protein concentration was determined by the method of Lowry *et al.* (14), using bovine serum albumin as a protein standard. Control incubations did not contain the NADPH-generating system; nonenzymatic binding in control incubations was always less than 15% of the binding in the experimental incubations.

To measure the rate of deethylation of phenacetin, 200 μl of the supernatant obtained after precipitation of the protein with methanol were spotted on a 5 × 20 cm, 250-μm silica gel GF thin-layer plate, and 100 μg of nonradioactive acetaminophen were added as a marker. The chromatogram was then developed in ether. The spot containing acetaminophen (R_F 0.25) was scraped directly into a counting vial, and the amount of [^{3}H]acetaminophen formed was quantitated by liquid scintillation spectrometry.

Glutathione trapping experiments. All incubation mixtures (150 ml) contained hamster liver microsomes (3 mg of protein per milliliter), glutathione (1 mM), NADP (0.67 mM), glucose 6-phosphate (3 mM), glucose 6-phosphate dehydrogenase (200 units), magnesium chloride (3 mM), and substrate (1 mM) and were exposed to the same $^{18}O_2$ atmosphere. One incubation mixture contained [^{3}H]acetaminophen (1 mM), another contained [^{3}H]phenacetin (1 mM), and a third contained acetanilide (1 mM). The acetanilide incubation was included to determine the maximal incorporation of ^{18}O during formation of the *p*-hydroxylated product (acetaminophen). The incubation mixtures were contained in 500-ml suction flasks connected to one another by heavy rubber tubing. The system was connected by a tube to a vial containing 250 ml of $^{18}O_2$ gas. It was also connected by rubber tubing to a nitrogen tank and a water vacuum. While immersed in an ice bath, the incubation mixtures were evacuated seven times, and the atmosphere was replaced with a nitrogen atmosphere each time. After a final evacuation, the seal to the $^{18}O_2$ gas was broken and the $^{18}O_2$ atmosphere was allowed to distribute over the three incubations, followed by nitrogen gas to equalize the pressure. The calculated atmospheric concentrations were 14% $^{18}O_2$ and 86% N_2. All incubation mixtures were incubated simultaneously in a shaking water bath at 37° for 20 min. Subsequently each reaction was terminated with 100 ml of cold acetone, and the protein was removed by centrifugation. Acetone was removed by flash evaporation, and the aqueous phases (approximately 150 ml) were extracted four times with 2 volumes each of ethyl acetate. The organic extract from the acetanilide incubation was evaporated under a stream of nitrogen, and the *p*-hydroxylated product (acetaminophen) was separated on two 20 × 20 cm, 2000-μm silica gel GF plates (ether solvent). The aqueous phases from the acetaminophen and phenacetin incubation mixtures were freeze-dried and subsequently dissolved in a minimal amount of water. One half of each sample was used to isolate the glutathione conjugates. The material from each incubation was spotted on six 20 × 20 cm, 1000-μm Avicel F plates (solvent, 1-propranol-H_2O, 70:30). An area corresponding to an R_F of 0.44–0.80 was removed and extracted with 100 ml of water, and the aqueous phase was freeze-dried. This material was further purified on six more Avicel F plates with the same solvent. This time the glutathione conjugate gave a sharp peak with an R_F of 0.58. The conjugates were subsequently eluted from the Avicel with water, freeze-dried, and treated with Raney nickel in methanol to reductively cleave the sulfur from the ring (15, 16). This procedure generated acetaminophen in both cases. However, the procedure did not remove an ethyl group from phenacetin or exchange the ^{18}O in acetaminophen, as determined by control experiments. The acetaminophen isolated from both conjugates was purified on 5 × 20 cm, 250-μm silica gel GF plates with ether as the solvent. The ether solvent material at R_F 0.25 was eluted from the silica gel with glass-distilled ethyl acetate. In control incubations, which did not contain the NADPH-generating system, the corresponding metabolites were absent.

Mass spectrometry. The electron impact mass spectra of these metabolites were de-

termined by direct probe insertion of samples of the isolated product from each incubation on a VG Micro Mass 16F instrument (accelerating voltage, 4 kV; electron energy, 70 eV; ionizing current, 100 μamp; ion source pressure, 2×10^{-7} torr; ion source temperature, 180°). Several spectra were run for each sample, and peak heights were determined. Standard procedures were followed in substracting isotope contributions from the preceding two peaks (P + 1 and P + 2) of peaks at *m/e* 109, 111, 151, and 153.

RESULTS

Incubation of [^{3}H]phenacetin with hamster liver microsomes and NADPH yielded a metabolite that was irreversibly bound to the microsomal macromolecules. This binding apparently was covalent, since repeated extractions with a variety of organic solvents failed to separate this metabolite from the macromolecules (2, 3). In addition, when the radiolabeled macromolecules were digested by Pronase and analyzed by thin-layer chromatography, the metabolite was inseparable from the amino acid residues. Furthermore, inclusion of the alternative nucleophile, glutathione, decreased the covalent binding of the metabolite by forming a covalent glutathione-metabolite adduct.

The covalent binding of phenacetin probably did not occur simply through activation of acetaminophen, the primary metabolite of phenacetin. If this were the mechanism, a lag phase in the covalent binding would have been expected as the acetaminophen concentration increased toward the K_m (one-half V_{max}). But no lag phase was observed; instead, the rate of formation of the arylating metabolite was constant with time for at least 15 min (Fig. 1A). The rate of the covalent binding reaction also was proportional to microsomal protein (enzyme) concentration up to 2.0 mg/ml (Fig. 1B).

The possible involvement of a cytochrome P-450 mixed-function oxidase in the formation of this reactive metabolite of phenacetin was studied (Table 1). In the complete system, which contained microsomes, [^{3}H]phenacetin, and an NADPH-generating system, 5.94 nmoles of metabolite were covalently bound to microsomes. Omission of the NADPH-generating system or boiling the enzyme for 10 min decreased the covalent binding to less than 15% of that obtained with the complete system. Replacement of the oxygen atmosphere with nitrogen or inclusion of a CO–O_2 (9:1) atmosphere decreased the covalent binding. In another experiment the rate of the covalent binding of phenacetin pro-

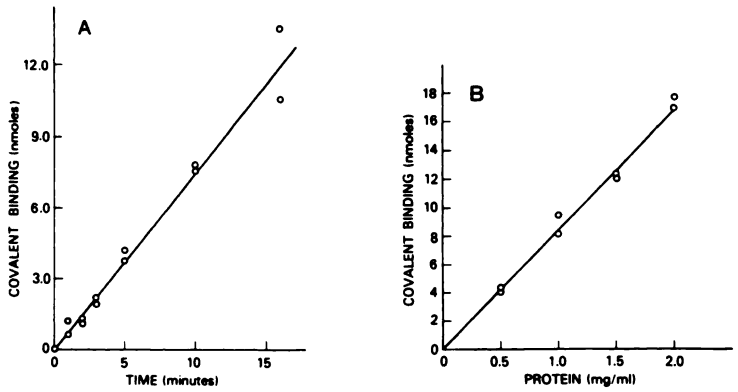

FIG. 1. *Covalent binding of phenacetin*

A. Time course. [^{3}H]Phenacetin (3 μmoles) was incubated with hamster liver microsomes (6 mg) and NADPH-generating system in a total volume of 3 ml for the indicated time periods.

B. Effect of protein (enzyme) concentration. [^{3}H]Phenacetin (3 μmoles) was incubated with the indicated concentration of hamster liver microsomes (a different preparation from that used in Fig. 1A) and an NADPH-generating system in a total volume of 3 ml for 10 min. Covalent binding is expressed as total nanomoles bound per incubation mixture.

TABLE 1

Effect of incubation conditions on covalent binding of phenacetin by hamster liver microsomes

The complete incubation mixture (3 ml) contained hamster liver microsomes (6 mg), [^{3}H]-phenacetin (3 μmoles), an NADPH-generating system, and an air atmosphere. In the heat treatment experiments the microsomes were heated to 90° for 10 min prior to use. In the N_2, N_2-O_2, and CO-O_2 experiments the air atmosphere was replaced by repeated evacuation and flushing with the appropriate atmosphere. Each mixture was incubated in triplicate for 10 min.

Conditions	Covalent binding[a]	Inhibition
	nmoles	%
Complete incubation	5.94 ± 0.30	
−NADPH	0.90 ± 0.06	85
Heat-treated microsomes	0.82 ± 0.12	86
−O_2 (100% N_2)	2.34 ± 0.06	61
+CO-O_2 (9:1)	3.24 ± 0.06	45
+N_2-O_2 (9:1)	4.28 ± 0.18	28
+NaF (0.1 M)[b]	4.72 ± 0.12	21
+Acetaminophen (0.05 mM)	6.60 ± 0.06	0

[a] Covalent binding is expressed as total nanomoles per incubation mixture.

[b] In a control experiment the covalent binding of acetaminophen was increased by 98% by this concentration of sodium fluoride.

ceeded twice as fast with NADPH as with NADH (data not shown). These data suggest the involvement of a cytochrome P-450 mixed-function oxidase in the covalent binding of [^{3}H]phenacetin. Table 1 also shows that sodium fluoride did not stimulate the covalent binding, as it did with acetaminophen. The inclusion of low concentrations of unlabeled acetaminophen (0.05 mM) had no effect on the covalent binding of phenacetin.

Administration of phenobarbital or 3-methylcholanthrene, which induce various cytochrome P-450 mixed-function oxidases, resulted in different patterns of induction in the formation of the reactive metabolites of acetaminophen and phenacetin (Table 2). As shown previously (5), treatment of hamsters with 3-methylcholanthrene increased the V_{max} for the formation of the reactive metabolite of acetaminophen and treatment with phenobarbital did not alter the V_{max}. In contrast, treatment of hamsters with 3-methylcholanthrene did not increase the V_{max} of covalent binding of phenacetin, whereas treatment of hamsters with phenobarbital slightly increased it. Piperonyl butoxide decreased the V_{max} for covalent binding of both phenacetin and acetaminophen (data not shown).

In contrast to the different inductive effects seen with 3-methylcholanthrene and phenobarbital on the covalent binding of acetaminophen and phenacetin, both 3-methylcholanthrene and phenobarbital increased the rate of deethylation of phenacetin. The V_{max} for deethylation in untreated animals was 23 nmoles/10 min/mg of protein (K_m, 0.05 mM); in phenobarbital-treated animals, 45 nmoles/10 min/mg of protein (K_m, 0.10 mM); and in 3-methylcholanthrene-treated animals, 55 nmoles/10 min/mg of protein (K_m, 0.05 mM).

The possible involvement of *N*-hydroxyphenacetin in the covalent binding of phenacetin was investigated by incubation of [^{14}C]*N*-hydroxyphenacetin with microsomes (Fig. 2). *N*-Hydroxyphenacetin did not spontaneously bind covalently to mi-

TABLE 2

Effect of inducers of cytochrome P-450 on covalent binding of acetaminophen and phenacetin

Animals (four in each group) were treated as described in MATERIALS AND METHODS. The assays were performed as described in MATERIALS AND METHODS, except that the substrate concentration was varied between 0.05 and 1.0 mM (five different concentrations were used, and each mixture was incubated in duplicate). The same microsomal preparations were used to compare the treatment effects on the covalent binding of phenacetin and acetaminophen. The K_m and V_{max} values were determined from a plot of S/v vs. S with the aid of a computer program.

Substate and treatment of animals	V_{max}	K_m
	nmoles/10 min/mg	*mM*
Acetaminophen		
No treatment	1.30 ± 0.08	0.13
3-Methylcholanthrene	2.25 ± 0.08	0.18
Phenobarbital	1.33 ± 0.08	0.06
Phenacetin		
No treatment	1.76 ± 0.07	0.27
3-Methylcholanthrene	1.37 ± 0.07	0.27
Phenobarbital	2.03 ± 0.09	0.27

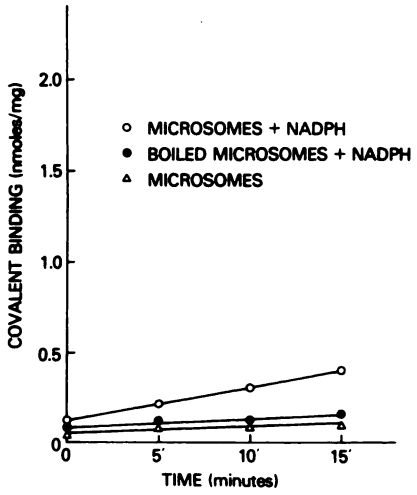

FIG. 2. *Covalent binding of [^{14}C]N-hydroxyphenacetin*

[^{14}C]*N*-Hydroxyphenacetin (1.5 μmoles) in a total volume of 3.0 ml was incubated for the indicated time periods with either hamster liver microsomes plus an NADPH-generating system, boiled hamster liver microsomes (90° for 10 min) plus an NADPH-generating system, or hamster liver microsomes without an NADPH-generating system. Each point is the average of two experiments.

crosomes during a 20-min incubation at 37°. A small amount of binding was observed when an NADPH-generating system was included in the microsomal incubation mixture, but the rate was approximately 10% of that observed with phenacetin (Fig. 1A). It therefore seems unlikely that *N*-hydroxyphenacetin is the major metabolite of phenacetin which is covalently bound to hamster liver microsomes.

To gain insight into the mechanism of activation of phenacetin and acetaminophen, and to characterize the nature of their covalently bound species, the reactive metabolites were trapped by including 1 mM glutathione in the incubation mixtures. Glutathione decreased the covalent binding of acetaminophen by 90%, and that of phenacetin by 75%; a glutathione conjugate was formed in each case. Analysis of these glutathione conjugates by thin-layer chromatography (Avicel F, 250 μm; solvent, 1-propranol–H_2O, 70:30) showed them to be indistinguishable (R_F 0.58). When the glutathione conjugates of acetaminophen and phenacetin were isolated from thin-layer plates and treated with Raney nickel in methanol to reductively cleave the glutathione from the aromatic ring, acetaminophen was isolated from each conjugate. In control experiments, however, treatment of phenacetin with Raney nickel in methanol did not produce acetaminophen.

We next examined these metabolites by mass spectrometry after their formation in mixtures incubated under an $^{18}O_2$ atmosphere. If phenacetin or acetaminophen were activated to an arylating metabolite by 3,4-epoxidation, with a subsequent rearrangement to acetimidoquinone, ^{18}O would be incorporated into the *para* position of the metabolite. If *N*-hydroxylation were the mechanism of activation of phenacetin or acetaminophen, ^{18}O would not be incorporated into the metabolite. In addition, an acetanilide incubation mixture was included as a control to determine the relative incorporation of ^{18}O into the *p*-hydroxylated acetaminophen product. The acetaminophen formed from acetanilide was then compared with the acetaminophen formed after Raney nickel cleavage of the glutathione conjugates from the acetaminophen and phenacetin incubation mixtures.

Figure 3A shows the mass spectrum of the metabolite obtained after Raney nickel treatment of the glutathione conjugate from the acetaminophen-glutathione incubation mixture. The spectrum shows a parent peak at *m/e* 151, corresponding to the molecular weight of ^{16}O-containing acetaminophen. The absence of a significant parent peak at *m/e* 153 [^{18}O acetaminophen] shows that little ^{18}O was incorporated during metabolic activation of acetaminophen to a chemically reactive electrophile by microsomal cytochrome P-450. The presence of the base peak at *m/e* 109, corresponding to the loss of ketene (^{16}M-42), and the absence of a significant peak at *m/e* 111 (^{18}M-42) confirm this observation.

Figure 3B shows the mass spectrum of the metabolite obtained after Raney nickel treatment of the glutathione conjugate isolated from the phenacetin-glutathione incubation mixture. The compound had par-

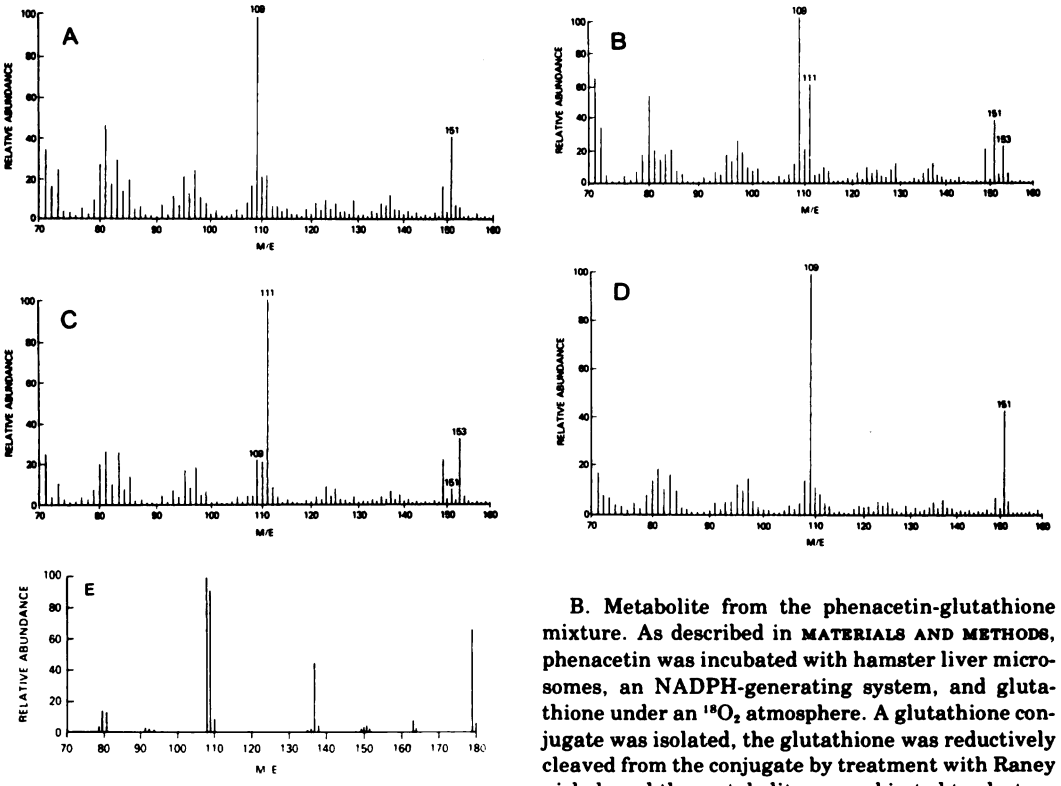

FIG. 3. *Mass spectra*

A. Metabolite from the acetaminophen-glutathione incubation mixture. As described in MATERIALS AND METHODS, acetaminophen was incubated with hamster liver microsomes, an NADPH-generating system, and glutathione under an $^{18}O_2$ atmosphere. A glutathione conjugate was isolated, the glutathione was reductively cleaved from the conjugate by treatment with Raney nickel, and the metabolite was subjected to electron impact mass spectrometry.

B. Metabolite from the phenacetin-glutathione mixture. As described in MATERIALS AND METHODS, phenacetin was incubated with hamster liver microsomes, an NADPH-generating system, and glutathione under an $^{18}O_2$ atmosphere. A glutathione conjugate was isolated, the glutathione was reductively cleaved from the conjugate by treatment with Raney nickel, and the metabolite was subjected to electron impact mass spectrometry.

C. Acetaminophen from the acetanilide incubation mixture. As described in MATERIALS AND METHODS, acetanilide was incubated with hamster liver microsomes and an NADPH-generating system under an $^{18}O_2$ atmosphere. The *p*-hydroxylated product was isolated and subjected to electron impact mass spectrometry.

D. Acetaminophen (commercial).

E. Phenacetin (commercial).

ent peaks at *m/e* 151 and 153, corresponding to ^{16}O acetaminophen and ^{18}O acetaminophen, respectively. The peak height ratio of 3:2 indicates 40% incorporation of ^{18}O into the acetaminophen metabolite. The ratio of the heights of the base peaks at *m/e* 109 and 111 (M-42) was the same as the ratio of the *m/e* 151 and 153 peaks. Thus atmospheric oxygen was incorporated into the arylating metabolite formed from phenacetin.

The mass spectrum of the *p*-hydroxylated metabolite (acetaminophen) formed from acetanilide is shown in Fig. 3C. The compound has parent peaks at *m/e* 151 and 153 in a ratio of 1:4, indicating 80% incorporation of ^{18}O into the acetanilide molecule. The ratio of the peaks at *m/e* 109 and 111 also indicates 80% incorporation of ^{18}O into the ring. In other experiments we found much greater incorporation of ^{18}O into acetanilide, depending on the success with which atmospheric $^{16}O_2$ was removed from incubation vials during the evacuation and nitrogen flushing procedures. In the present experiments, therefore, it seems likely that the $^{18}O_2$ constituted only 80% of the total oxygen atmosphere. Previous workers have shown that the oxygen incorporated into acetanilide during hy-

droxylation is derived from molecular oxygen (17, 18). In a repeat of this experiment, 96% ^{18}O was incorporated into acetanilide, 50% ^{18}O was incorporated into the phenacetin metabolite, and no ^{18}O was incorporated into the acetaminophen metabolite, as indicated by the peak ratios *m/e* 151:153 and *m/e* 109:111. These spectra, however, were contaminated with phthalic esters from the thin-layer plates and are not shown.

Figure 3D shows a mass spectrum of commercial acetaminophen, and Fig. 3E shows a mass spectrum of commercial phenacetin. These spectra are included for comparative purposes.

DISCUSSION

The present results show that a reactive metabolite of phenacetin is generated by a microsomal enzyme(s) *in vitro*. The mechanism of binding of phenacetin cannot be simply an activation of the major metabolite, acetaminophen, which is in equilibrium with the aqueous medium. The velocity of covalent binding of phenacetin was greater than that of acetaminophen in untreated hamsters (Table 2), yet only a fraction of phenacetin was converted to acetaminophen *in vitro* (138 nmoles at the end of the 10-min incubation period). Moreover, added nonradioactive acetaminophen should have inhibited the reaction by about 20% but did not affect the covalent binding of phenacetin (Table 1).

In addition, the other experiments show that the phenacetin metabolite is formed by a mechanism different from the generation of the reactive metabolite from acetaminophen. First, the effects of inducers on the formation of the reactive metabolites of acetaminophen and phenacetin differed (Table 2). Treatment with 3-methylcholanthrene increased the V_{max} for covalent binding of acetaminophen but decreased the V_{max} for covalent binding of phenacetin. Phenobarbital treatment, however, increased the V_{max} for covalent binding of phenacetin but did not significantly alter the V_{max} for covalent binding of acetaminophen.

Second, the effects of inducers on the covalent binding reaction were also different from their effects on the deethylation of phenacetin to acetaminophen; both phenobarbital and 3-methylcholanthrene treatment increased the rate of deethylation of phenacetin.

Third, sodium fluoride showed different effects on acetaminophen and phenacetin covalent binding (Table 1). This compound increased the covalent binding of acetaminophen and decreased the covalent binding of phenacetin.

Fourth, glutathione trapping experiments in the presence of an $^{18}O_2$ atmosphere showed that the phenacetin metabolite had become deethylated during activation and had undergone approximately 50% incorporation of atmospheric ^{18}O into the *para* position relative to the incorporation of ^{18}O during hydroxylation of acetanilide (Fig. 3B). The metabolite isolated from the acetaminophen-glutathione incubation mixture, however, showed almost no incorporation of atmospheric ^{18}O into the metabolite (Fig. 3A).

The finding that atmospheric ^{18}O was not incorporated into the metabolite from the acetaminophen-glutathione incubation mixture (Fig. 3A) is consistent with our previous postulate that *N*-hydroxylation leads to the production of the reactive metabolite of acetaminophen (1–6, 8). This mechanism is shown in Fig. 4A. Once formed, *N*-hydroxyacetaminophen presumably is dehydrated, losing $H_2{}^{18}O$ and forming acetimidoquinone, which then covalently combines with tissue macromolecules or glutathione (Fig. 4A).

With phenacetin, however, the incorporation of ^{18}O into the metabolite isolated from the incubation mixture of microsomes, phenacetin, and glutathione (Fig. 3B) is evidence for a different mechanism, possibly involving 3,4-epoxidation. The 50% incorporation of atmospheric ^{18}O into the phenacetin metabolite relative to the nearly complete incorporation of ^{18}O during the *p*-hydroxylation of acetanilide, however, is puzzling. If 3,4-epoxidation were the mechanism of activation of phenacetin, followed by rearrangement to yield the known arylating acetimidoquinone (7), the ethoxy group would be expected to be lost, resulting in 100% incorporation of atmospheric ^{18}O into the metabolite. To explain the 50% incorporation of atmospheric

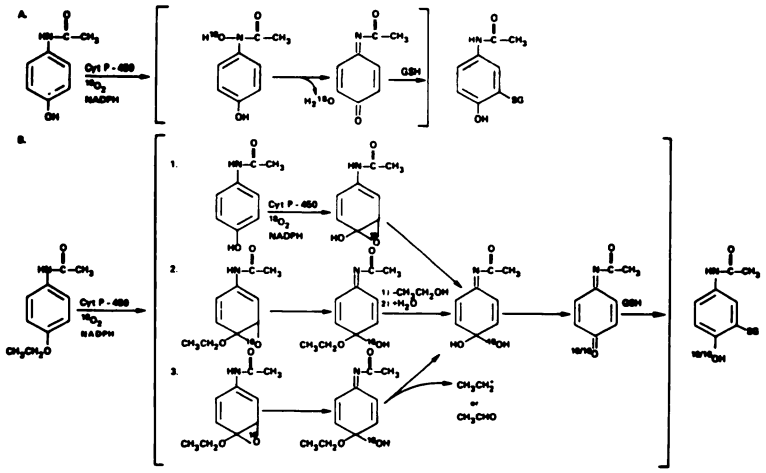

FIG. 4.

A. Proposed mechanism for activation of acetaminophen to an arylating metabolite.

B. Three possible mechanisms for activation of phenacetin to an arylating metabolite.

^{18}O it is necessary to postulate the loss of an ethyl group by some unknown mechanism, possibly a carbonium ion or the exchange of the ethoxy group with water. Both these mechanisms would represent unusual modes of deethylation. Another possible mechanism would be deethylation of phenacetin, followed by 3,4-epoxidation of the acetaminophen formed. This mechanism seems unlikely, since exogenous acetaminophen is apparently not activated in this manner. These three possible mechanisms are shown in Fig. 4B. Whatever the mechanism for the formation of a reactive metabolite of phenacetin may be, the 50% incorporation of ^{18}O into the conjugate requires that the carbon atom in the *para* position of phenacetin become tetrahedral and bind ^{16}O and ^{18}O in an equivalent manner. Experiments are in progress to elucidate the mechanism of reactive metabolite formation from phenacetin and to determine the importance of such a mechanism(s) in causing the various toxicities elicited by phenacetin.

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