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Metabolism of [p-^{18}O]-Phenacetin: The Mechanism of Activation of Phenacetin to Reactive Metabolites in Hamsters

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SUMMARY

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There are three different pathways by which phenacetin can be converted by liver enzymes to electrophilic reactive metabolites which may combine with glutathione to form an acetaminophen-glutathione conjugate: 1) Phenacetin is first deethylated to acetaminophen which is subsequently activated by cytochrome P-450. 2) Phenacetin is activated via an intermediate, possibly phenacetin-3,4-epoxide. 3) Phenacetin is first converted to N-hydroxyphenacetin, then activated by sulfation or glucuronidation. This paper shows that these three pathways can be distinguished by the disposition of ^{18}O in the para position of the ring: 1) When [p-^{18}O]-acetaminophen was activated *in vitro* and *in vivo* there was a negligible loss of ^{18}O in the respective acetaminophen-glutathione conjugate and the urinary acetaminophen-mercapturic acid. 2) When [p-^{18}O]-phenacetin was activated *in vitro* there was a 50% loss of ^{18}O in the acetaminophen-glutathione conjugate. 3) When N-hydroxyphenacetin glucuronide was incubated with $H_2{}^{18}O$ there was a quantitative incorporation of ^{18}O into the acetaminophen-glutathione conjugate. When N-hydroxyacetaminophen, the proposed intermediate in pathway 1, was incubated with glutathione and $H_2{}^{18}O$ there was no incorporation of ^{18}O into the acetaminophen-glutathione conjugate. The relative *in vivo* importance of the three pathways was investigated by i.p. injection of 50 mg/kg of [p-^{18}O]-phenacetin into two hamsters. The urinary acetaminophen mercapturic acid showed approximately a 10% loss of ^{18}O label, indicating that deethylation of phenacetin to acetaminophen followed by activation of acetaminophen, pathway 1, is the predominant pathway *in vivo*.

INTRODUCTION

In an effort to develop an animal model for renal damage, it was discovered that large doses of phenacetin causes centrilobular hepatic necrosis in hamsters, probably through the formation of a chemically reactive metabolite (1, 2).

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Studies *in vitro* have shown that phenacetin may be converted to chemically reactive metabolites through at least three pathways. In one pathway, phenacetin undergoes an oxidative deethylation to form acetaminophen (3, 4) which, in turn, is converted to a chemically reactive metabolite that may cause liver necrosis (5–8). In the second pathway, liver microsomes convert phenacetin directly to an arylating metab-

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olite that in the presence of GSH[2] may be trapped as an acetaminophen-GSH conjugate (9). In contrast to acetaminophen where no oxygen is incorporated into the conjugate, 50% of the ^{18}O in the phenolic group of the conjugate arising from phenacetin originates from atmospheric ^{18}O. In the third pathway, liver microsomes convert phenacetin to N-hydroxyphenacetin (10). In turn, N-hydroxyphenacetin is converted to N-O-sulfate and N-O-glucuronide conjugates that decompose to chemically reactive metabolites which may be trapped as a GSH conjugate of acetaminophen (11, 12).

The present study was undertaken to determine the origin of the phenolic ^{18}O of the acetaminophen-GSH conjugates which are formed from phenacetin by the three pathways. The results not only clarified the mechanisms by which reactive metabolites are formed from phenacetin but also raised the possibility that the relative importance of the three pathways might be determined by administering phenacetin labeled with ^{18}O in the ethoxy group to hamsters and by measuring the retention of ^{18}O in the acetaminophen mercapturic acid excreted into urine.

MATERIALS AND METHODS

[G-^{3}H]-Phenacetin, [G-^{3}H]-acetaminophen and [1-^{14}C]-acetyl chloride were purchased from New England Nuclear Corp. (Boston, Mass.). [1-^{14}C]-N-Hydroxyphenacetin was synthesized by zinc reduction of p-nitrophentole followed by acetylation with [1-^{14}C]-acetyl chloride as previously described (10). [^{18}O]-Phenol (82.5% ^{18}O) and [^{18}O]-water were purchased from Miles Labs, Inc. (Elkhart, IN). N-Hydroxyacetaminophen was the generous gift of Dr. I. C. Calder (University of Melbourne, Melbourne, Australia). Pyridine nucleotides, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Calbiochem (San Diego, CA). Activated Raney nickel was purchased from K and K. Thin layer chromatography plates were a product of Analtech (Newark, DE). Male golden Syrian hamsters (65 g) were obtained from the Animal Production Unit at the N.I.H. Animals had free access to food and water. All reagents were of the finest grade commercially available.

Synthesis of [p-^{18}O]-phenacetin. [^{18}O]-Phenol was converted to [p-^{18}O]-nitrosophenol by a modification of a technique described by Wassein (13). [^{18}O]-Phenol (0.50 g) was dissolved in 11.3 ml water to which was added 0.24 g of sodium hydroxide, 0.40 ml ammonium hydroxide and 0.46 g of sodium nitrite. The solution was cooled to 5° and 1.25 g of sulfuric acid in 3.4 ml of water was added dropwise over a 1 hr period. The crude precipitate was filtered and the filtrate extracted with ethyl acetate (2 vol). The ethyl acetate phase was removed on a rotary evaporator and the residue added to the precipitate. The crude [p-^{18}O]-nitrosophenol was dissolved in ethanol (30 ml) and 2 ml of concentrated HCl added. Raney nickel (10 g wet weight) was added to the ethanol solution and refluxed for 1 hr. The solution was then filtered, the filtrate evaporated on a rotary evaporator to dryness and the residue added to the precipitate. The crude [p-^{18}O]-aminophenol was subsequently acetylated by modification of a procedure described by Vogel (14). The crude [p-^{18}O]-aminophenol was dissolved in 7 ml of H_2O to which was added 1.75 ml acetic anhydride. The solution was stirred for 30 min and then another 1 ml of acetic anhydride was added. After being stirred for an additional 30 min, the solution was adjusted to pH 7.0 with 10 N NaOH and extracted with two 100 ml aliquots of ethyl acetate. The ethyl acetate solution was evaporated on a rotary evaporator to dryness to give 450 mg of [p-^{18}O]-acetaminophen for a 56% overall yield of crude product. Before use, a portion of the acetaminophen was recrystallized from hot water according to the method of Vogel (14). The compound showed a single spot on silica gel thin layer chromatography (solvent ether) (R_F 0.25). Mass spectral analysis showed the compound contained 48% ^{18}O.

[p-^{18}O]-Phenacetin was synthesized by ethylation of the crude [p-^{18}O]-acetamino-

[2] The abbreviations used are GSH, glutathione and UDPGA, uridine diphosphoglucuronic acid.

phen (250 mg) with ethyl iodide as described by Vogel (14) (yield 220 mg crude product). After recrystallization from ether, 113 mg of [p-^{18}O[-phenacetin was obtained and gave a single spot as determined by thin layer chromatography on silica gel (solvent ether) (R_F 0.5). The percentage ^{18}O was identical to the [p-^{18}O]-acetaminophen.

GSH trapping experiments. GSH conjugates were formed by procedures similar to those previously described (9). The incubation mixtures (150 ml) contained hamster liver microsomes (3 mg protein per ml), GSH (1 mM), NADP (0.67 mM), glucose-6-phosphate (3 mM), glucose-6-phosphate dehydrogenase (200 units), $MgCl_2$ (3 mM) and substrate (1 mM). One incubation mixture contained [p-^{18}O]-phenacetin (1 mM) and another incubation mixture contained [p-^{18}O]-acetaminophen (1 mM). Both mixtures were incubated in a shaking water bath at 37° for 20 min. Subsequently each reaction was terminated with 100 ml of cold acetone, and the precipitated protein separated from the aqueous phase by centrifugation. Acetone was removed from the aqueous phase on a rotary evaporator and the aqueous phases were extracted four times with 2 vol of ethyl acetate. The aqueous phases were freeze-dried. The residues were dissolved in a minimal amount of water and one-half of each sample was spotted on six 20 × 20 cm, 1000 μ Avicel F plates (solvent, 1-propranol-H_2O, 70:30). The Avicel containing the GSH conjugates (R_F 0.58) were scraped from the plates and extracted with 100 ml of water. After freeze-drying the extract, the residues were further purified on two 20 × 20 cm 1000 μ Avicel F plates with the same solvent. The GSH conjugates were subsequently eluted from the Avicel with water, freeze-dried and treated with Raney nickel in methanol for 30 min to cleave reductively the sulfur from the ring. The Raney nickel was removed by filtration and the methanol removed by evaporation under a stream of nitrogen. The acetaminophen isolated from the conjugates was purified on a 5 × 20 cm, 250 μ silica gel GF plate with ether as the solvent (R_F 0.25). The acetaminophen was removed from the silica gel by extraction with chromatographic grade ethyl acetate.

Acetaminophen and its GSH conjugate were isolated after the nonenzymatic breakdown of phenacetin N-O-glucuronide in $H_2{}^{18}O$. In this experiment 10 μmoles of phenacetin N-O-glucuronide, formed from N-hydroxyphenacetin and UDPGA in the presence of liver microsomes and isolated as previously described (12), was incubated for 45 hr at 37° with 75 μmoles of GSH in 0.05 M Tris HCl buffer, pH 7.4, containing 22% $H_2{}^{18}O$ (3 ml). The acetaminophen formed during the breakdown of phenacetin N-O-glucuronide was extracted from the solution with ethyl acetate and isolated on a 20 × 20 cm, 1000 μ silica gel GF thin layer plate (solvent, ether). The acetaminophen-GSH conjugate which remained in the aqueous phase after extraction of the incubation mixture with ethyl acetate was isolated by thin layer chromatography, treated with Raney nickel and the acetaminophen isolated as described above.

An acetaminophen-GSH conjugate was also isolated from the nonenzymatic breakdown of N-hydroxyacetaminophen in $H_2{}^{18}O$. In this experiment 21 μmoles of N-hydroxyacetaminophen was incubated with 25 μmoles of GSH, 100 μmoles of potassium phosphate, pH 7.4, and 50% $H_2{}^{18}O$ in a total vol of 1 ml at 37° overnight (15 hr). The mixture was extracted with ethyl acetate and the acetaminophen in the extract isolated on a 20 × 20 cm, 1000 μ silica gel GF plate (solvent, ether). The acetaminophen-GSH conjugate was isolated on a 20 × 20 cm, 1000 μ Avicel F thin layer plate (solvent 1-propanol/H_2O, 70:30) (R_F 0.58). The conjugate was reductively cleaved to form acetaminophen with Raney nickel as previously described. The acetaminophen was subsequently purified on a 20 × 20 cm, 1000 μ silica gel GF thin layer plate (solvent, ether) and extracted from the plate with chromatographic grade ethyl acetate.

Isolation of mercapturic acid. Two male 65 g hamsters were given 3.25 mg (50 mg/kg, i.p.) of generally tritiated (80.6 μCi) [p-^{18}O]-labeled phenacetin dissolved in 0.15 ml Tween 80. Two male 65 g hamsters were similarly given i.p. 3.25 mg of generally tritiated (38.8 μCi) [p-^{18}O]-acetaminophen (50 mg/kg) in 0.15 ml Tween 80. Each hamster was kept in an individual metabolic

cage and the urine collected for 24 hr over dry ice. Each urine was extracted 3 times with 20 ml aliquots of ethyl acetate. The pH of the urines were then decreased to pH 1.0 by addition of concentrated HCl and the urines were again extracted three times with 20 ml aliquots of ethyl acetate. The ethyl acetate extracts from each urine were evaporated on a rotary evaporator. Approximately one third of the total of each urine was added to a 20 × 20 cm, 1000 μ Avicel F plate and the chromatograph was developed with a solvent of 1-propranol:0.4 M ammonium hydroxide, 80:20, as previously described (15). The mercapturic acids from each animal were then removed from the Avicel by extraction with water. The water was removed by lyophilization and the mercapturates were treated with Raney nickel as described above to reductively cleave the N-acetylcysteine from acetaminophen. After the Raney nickel treatment the acetaminophen was purified on a 5 × 20 silica gel GF thin layer chromatographic plate (solvent, ether) as described above.

Isolation of acetaminophen from glucuronide and sulfate conjugate. The pH of each acidified urine was adjusted to 5.0 with NaOH; 0.2 ml of a β-glucuronidase/arylsulfatase preparation from Helix pomatia (Boehringer Mannheim) was added and the solution incubated for 24 hr. Each urine was extracted three times with 20 ml aliquots of ethyl acetate and the acetaminophen from each sample was isolated by thin layer chromatography (20 × 20 cm, 1000 μ silica gel GF; solvent, ether).

Mass spectrometry. The electron impact mass spectra of the *in vitro* metabolites were determined as previously described by direct probe insertion of samples of the isolated product into a VG Micro Mass 16F instrument (accelerating voltage, 4 kV; electron energy, 70 eV; ionizing current 100 μ amp; ion source pressure, 2×10^{-7} Torr; ion source temperature, 180°) (9). Several spectra were obtained from each sample, and the peak heights of the ^{18}O and ^{16}O isotopes were determined.

The samples isolated from hamster urinary metabolites were methylated with diazomethane and analyzed by gas chromatography-chemical ionization mass spectrometry and selected ion monitoring of m/e 168 and m/e 166 by the method of Garland *et al.* (16).

RESULTS

In vitro studies. When [p-^{18}O]-acetaminophen was incubated with hamster liver microsomes, GSH and a NADPH-generating system as described in METHODS, an acetaminophen-GSH conjugate was isolated and treated with Raney nickel to cleave reductively to form acetaminophen. Figure 1A shows a mass spectrum of the isotopic mixture of [p-^{18}O] and [p-^{16}O]-acetaminophen that was used in the microsomal incubation mixtures. Based on the peak height ratios after subtracting ^{13}C contributions, acetaminophen contained 48% ^{18}O and 52% ^{16}O. The analysis was consistent for both the molecular ions at m/e 153 and 151, and the base peak ions at m/e 111 and 109 which correspond to a loss of the elements of ketene. Table 1 shows the ^{18}O content of the acetaminophen substrate and the acetaminophen-GSH conjugate

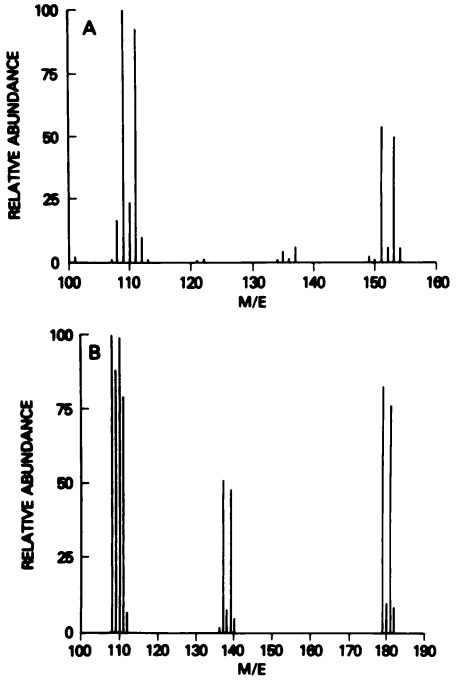

FIG. 1. *Mass spectra*
A) [p-^{18}O]-Acetaminophen. B) [p-^{18}O]-Phenacetin.

TABLE 1

^{18}O content of acetaminophen-GSH conjugates from [p-^{18}O]-acetaminophen and [p-^{18}O]-phenacetin microsomal incubation mixture

[p-^{18}O]-Acetaminophen and [p-^{18}O]-phenacetin were incubated with hamster liver microsomes, an NADPH generating system and GSH as described in MATERIALS AND METHODS. The acetaminophen-GSH conjugates were isolated as well as the acetaminophen metabolite from the [p-^{18}O]-phenacetin incubation mixture and the ^{18}O content determined by mass spectrometry. The mass spectral assay has a standard deviation of ±3%. The value for the acetaminophen-GSH conjugate from the [p-^{18}O]-phenacetin incubation mixture is an average of two mass spectral determinations.

Substrate	Metabolite	% ^{18}O
Acetaminophen		48
	Acetaminophen-GSH conjugate	45
Phenacetin		48
	Acetaminophen-GSH conjugate	23
	Acetaminophen metabolite	46

isolated from the incubation mixture. Calculations from several spectra showed that the metabolite contained 45% ^{18}O. These data indicate that there was only a minor loss, if any, of ^{18}O in the activation of acetaminophen to a reactive metabolite, since the standard deviation of precision for the method was ± 3%.

[p-^{18}O]-Phenacetin, was also incubated with hamster liver microsomes, GSH and a NADPH-generating system and a GSH conjugate was isolated and treated with Raney nickel as described in MATERIALS AND METHODS. Figure 1B shows a mass spectrum of the isotopic mixture of ^{18}O- and ^{16}O-phenacetin that was used in the incubation mixture. Based on an analysis of the molecular ions at m/e 181 and 179, and ions at 139 and 137 (which indicate the loss of ketene), the mixture contained 48% ^{18}O (Table 1). Moreover, there was an average of 23% ^{18}O in the resulting acetaminophen-GSH conjugate indicating there was a 50% loss of the ^{18}O during the activation of phenacetin to a reactive metabolite (Table 1). These data are consistent with the previous observation that there is a 50% incorporation of molecular $^{18}O_2$ into the reactive metabolite (9). The acetaminophen isolated from this incubation mixture (Table 1) contained 46% ^{18}O indicating an insignificant loss of ^{18}O since the standard deviation of precision for the method was ± 3%.

To determine the fate of the ^{18}O in its ethoxy group, N-hydroxyphenacetin glucuronide was incubated with GSH in the presence of $H_2{}^{18}O$ which was 22% ^{18}O as calculated by mixing $H_2{}^{18}O$ with a known amount of $H_2{}^{16}O$. The acetaminophen-GSH conjugate was isolated and treated with Raney nickel. A mass spectral analysis (Table 2) of the acetaminophen revealed that 24% of the phenolic oxygen was ^{18}O. Thus, all of the oxygen in the ethoxy group was replaced by oxygen from water.

Studies with N-hydroxyacetaminophen. It has been suggested that the chemically reactive metabolite of acetaminophen occurs through the formation of N-hydroxyacetaminophen followed by a spontaneous dehydration to yield acetylimidoquinone (1). In accord with this view, Calder *et al.* (17, 18) have synthesized N-hydroxyacetaminophen and found that it decreases the GSH in mouse liver and causes liver necrosis. Moreover, Andrews[3] showed *in vitro* that N-hydroxyacetaminophen decomposes to a species which reacts with GSH to form an acetaminophen conjugate. However, the finding that the ethoxy group of N-hydroxyphenacetin glucuronide exchanges with water, whereas the phenolic group of acetaminophen does not exchange with water during the formation of the conjugate, required further investigation. For this reason N-hydroxyacetaminophen was incubated with GSH in the presence of $H_2{}^{18}O$. From this incubation the acetaminophen and the acetaminophen-GSH conjugate were isolated and examined by mass spectrometry as described in MATERIALS AND METHODS for the incorporation of ^{18}O. As shown in table 3, there was no incorporation of ^{18}O into the acetaminophen and the acetaminophen-GSH conjugate.

In vivo studies. The *in vivo* metabolism of [p-^{18}O]-acetaminophen and [p-^{18}O]-phenacetin was studied in hamsters. Two hamsters were given 50 mg/kg of [p-^{18}O]-

[3] R. Andrews, personal communication.

TABLE 2

^{18}O Incorporation of $H_2{}^{18}O$ into acetaminophen and acetaminophen-GSH conjugate from decomposition of phenacetin N-O-glucuronide

As described in MATERIALS AND METHODS 10 μmoles of phenacetin N-O-glucuronide was incubated for 45 hr with 75 μmoles of GSH and 22% $H_2{}^{18}O$ in 0.05 M Tris HCl pH 7.4. The acetaminophen and acetaminophen-GSH conjugate were isolated. The ^{18}O content of each metabolite was determined by the ratio of the peaks at m/e 151:153 and m/e 109:111 after mass spectral analysis. The ^{18}O content of the water was calculated by addition of a known aliquot of $H_2{}^{18}O$ to unlabeled water.

Compound	% ^{18}O	% Incorporation of H_2O into metabolite
H_2O	22	—
Acetaminophen	24	100
Acetaminophen-GSH conjugate	24	100

TABLE 3

^{18}O Incorporation of $H_2{}^{18}O$ into acetaminophen and acetaminophen-GSH conjugate during decomposition of N-hydroxyacetaminophen

As described in MATERIALS AND METHODS, 21 μmoles of N-hydroxyacetaminophen was incubated with 25 μmoles of GSH and 59% $H_2{}^{18}O$ in 0.05 M Tris HCl, pH 7.4. The acetaminophen and acetaminophen-GSH conjugate were isolated. The ^{18}O content of each metabolite was determined by the ratio of the peaks at m/e 151:153 and m/e 109:111 after mass spectral analysis. The ^{18}O content of the water was calculated by addition of a known aliquot of $H_2{}^{18}O$ to unlabeled water.

Compound	% ^{18}O	% Incorporation of H_2O into metabolite
H_2O	59	—
Acetaminophen	0	0
Acetaminophen-GSH conjugate	0	0

acetaminophen and the urines collected for 24 hr. The acetaminophen mercapturates were isolated from the urine as described in MATERIALS AND METHODS and represented approximately 13% of the recovered radioactivity. The acetaminophen from the glucuronide and sulfate conjugates were also isolated as described in MATERIALS AND METHODS. For a more precise determination of the ^{18}O content in these metabolites, analysis was carried out by the mass spectral selected ion monitoring technique of Garland *et al.* (16). As shown in Table 4, there was only a minor loss of ^{18}O in the acetaminophen from the acetaminophen-mercapturic acid. These data are consistent with the *in vitro* data from Table 1 that in the conversion of acetaminophen to a reactive metabolite there is only a minor loss of the ^{18}O label. Two hamsters were also given 50 mg/kg of [p-^{18}O]-phenacetin. The urines were collected for 24 hr and acetaminophen-mercapturic acids isolated as described in MATERIALS AND METHODS. The radioactive mercapturic acids represented approximately 16% of the recovered radioactivity. The acetaminophen from the glucuronide and sulfate conjugates were also isolated as described in MATERIALS AND METHODS. As shown in Table 5, there was an average 3.4% loss of ^{18}O in the sulfate and glucuronide fraction and an average 9% loss of ^{18}O in the acetaminophen-mercapturic acid. The loss of ^{18}O in the acetaminophen recovered as sulfate or glucuronide may be significant since the precision of the method at the levels determined was ±1%. This may indicate that the minor losses of ^{18}O observed in the formation of acetaminophen from phenacetin *in vitro* (Table 1) might also be significant.

TABLE 4

^{18}O Content of acetaminophen and acetaminophen mercapturate after injection of [p-^{18}O]-acetaminophen into hamsters

Two hamsters were injected with 50 mg/kg [p-^{18}O]-acetaminophen and metabolites isolated as described in MATERIALS AND METHODS. The ^{18}O content of the acetaminophen and acetaminophen metabolites was determined by the method of Garland *et al.* (16). The ^{18}O content of the metabolites from each hamster is reported separately.

Sample	$^{18}O/^{16}O$	^{18}O in conjugate/^{18}O acetaminophen	Total loss of oxygen %
Injected acetaminophen	0.942	—	—
Acetaminophen from GSH and sulfate	1) 0.914	.985	1.5
	2) 0.915	.985	1.5
Acetaminophen from mercapturate	1) 0.900	.977	2.3
	2) 0.931	.994	0.6

TABLE 5

^{18}O Content of acetaminophen and acetaminophen mercapturate after injection of [p-^{18}O]-phenacetin into hamsters

Two hamsters were injected with 50 mg/kg [p-^{18}O]-phenacetin and the metabolites isolated as described in MATERIALS AND METHODS. The ^{18}O content of the phenacetin and the phenacetin metabolites was determined by the method of Garland *et al.* (16). The ^{18}O content of the metabolites from each hamster is reported separately.

Sample	$^{18}O/^{16}O$	^{18}O in conjugate/^{18}O in phenacetin	Total loss of oxygen %
Injected phenacetin	0.940	—	—
Acetaminophen from glucuronide and sulfate conjugates	1) 0.879	.965	3.6
	2) 0.884	.968	3.2
Acetaminophen from mercapturate	1) 0.805	.920	8.0
	2) 0.774	.900	10.0

DISCUSSION

Previous studies have revealed that liver preparations convert phenacetin to chemically reactive metabolites along three different pathways (3–12).

In one of the pathways (Pathway 3, Fig. 2C) phenacetin is converted to N-hydroxyphenacetin by cytochrome P-450 in hamster liver microsomes (10). N-Hydroxyphenacetin in turn may be converted to an N-O-sulfate conjugate by sulfurylase in the liver soluble fraction or to an N-O-glucuronide conjugate by UDP-glucuronyl transferase in liver microsomes (11). Both of the conjugates decompose to chemically reactive metabolites which lack the ethyl group of phenacetin (12). Although the N-O-sulfate conjugate is too unstable to isolate, the N-O-glucuronide has a half-life of about 8.7 hr in Tris buffer, pH 7.2 (12). During the decomposition of the N-O-glucuronide about 50% is converted directly to either phenacetin or the 2-hydroxyglucuronide of phenacetin, but the rest decomposes to a chemically reactive metabolite that is reduced to acetaminophen, hydrolyzed to acetamide and quinone and covalently combined with protein or GSH as acetaminophen derivatives (12). The present studies clarify the mechanism by which the ethoxy group of phenacetin is lost during

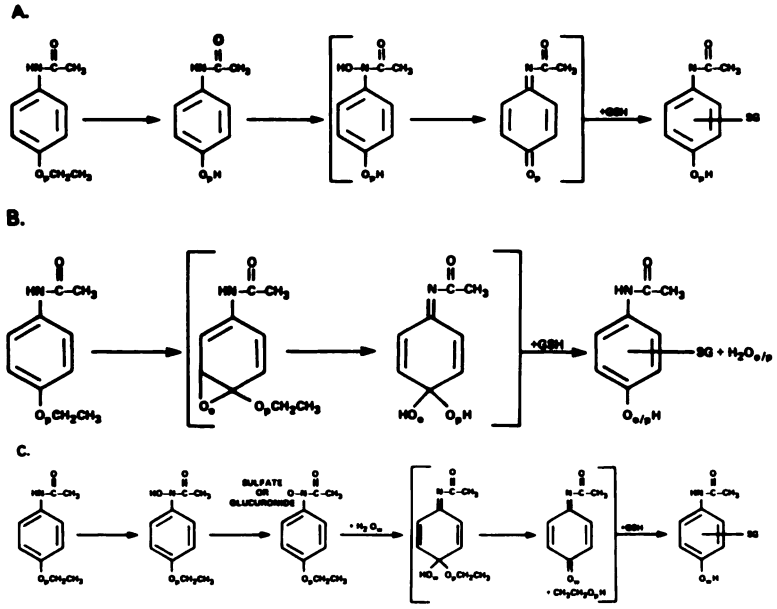

FIG. 2. *Three pathways along which liver preparations convert phenacetin to chemically reactive metabolites*

A) Pathway 1 for activation of phenacetin to a reactive metabolite. B) Pathway 2 for activation of phenacetin to a reactive metabolite. C) Pathway 3 for activation of phenacetin to a reactive metabolite. The compounds in brackets are proposed intermediates.

the formation of the chemically reactive metabolite. The ethoxy group is totally replaced by water in the reaction, possibly by the cleavage of the glucuronyl group from the nitrogen atom and the attack of an hydroxyl group at the para carbon to form a transient intermediate, the ethyl hemiketal of N-acetyl imidoquinone. Presumably the intermediate then decomposes to ethanol and N-acetylimidoquinone which in turn leads to acetaminophen and covalently bound acetaminophen.

In the second pathway (Pathway 2, Fig. 2B) phenacetin is converted by cytochrome P-450 in liver microsomes to an intermediate (presumably phenacetin-3,4-epoxide) which leads to the formation of another intermediate that combines with GSH to form the acetaminophen-GSH conjugate. Since 50% of the ^{18}O in the GSH conjugate originates from atmospheric ^{18}O (9), it seems likely that phenacetin-3,4-epoxide somehow further reacts to form the hydrate of N-acetylimidoquinone which retains equivalent hydroxy groups that arise from different sources of oxygen. The finding that 50% of the ^{18}O in the [p-^{18}O]-phenacetin is retained in the acetaminophen-GSH conjugate (Table 1) indicates that phenacetin rather than water serves as the source of the rest of the ^{18}O in the phenolic group.

In Pathway 1 (Fig. 2A), phenacetin is oxidatively deethylated (3, 4) presumably through the formation of an unstable hemiacetal that decomposes to acetaminophen and acetaldehyde (22, 23). In accord with this pathway, replacement of the α-hydrogens of the ethyl group by deuterium markedly decreases the rate of formation of acetaminophen (2, 24) which suggests that the breaking of the C-D bond is a rate-limiting step. Moreover, when [p-^{18}O]-phenacetin was incubated with liver microsomes from hamsters, virtually all of the ^{18}O in phenacetin was retained in acetaminophen (Table 1).

Studies with [p-^{18}O]-acetaminophen revealed that the phenolic ^{18}O of acetaminophen is also retained in the formation of the chemically reactive metabolite that is trapped as an acetaminophen-GSH conjugate (Table 1). Thus the pathway by which acetaminophen is converted to a chemically reactive metabolite clearly differs from the pathway by which phenacetin-N-O-glucuronide decomposes to a chemically reactive metabolite. At first the difference in pathways raised doubts that the formation of the chemically reactive metabolite of acetaminophen was mediated by N-hydroxyacetaminophen, but when N-hydroxyacetaminophen was allowed to decompose in buffer containing GSH and $H_2{}^{18}O$, no ^{18}O was found in either acetaminophen or the acetaminophen-GSH conjugate (Table 3). It is, therefore, evident that decomposition of N-hydroxyacetaminophen is not mediated by a hydrate of N-acetylimidoquinone analogous to the hemiketal of phenacetin proposed in Pathway 3, or the hydrate of acetylimidoquinone proposed in Pathway 2. Instead the proposed N-acetylimidoquinone may be initiated by ionization of the phenolic group of N-hydroxyacetaminophen as suggested by Calder *et al.* (17, 18).

Regardless of the validity of these interpretations of the data concerning the mechanisms of the three pathways by which phenacetin is converted to chemically reactive metabolites, the relative importance of the three pathways was established by injection of [p-^{18}O]-phenacetin into animals and measuring the retention of ^{18}O in the acetaminophen mercapturate in urine. If the reactive metabolite were formed *in vivo* mainly by way of acetaminophen (Pathway 1, Fig. 2A), nearly all of the ^{18}O would be retained in the acetaminophen mercapturic acid. Only 50% of the ^{18}O would be retained in the mercapturic acid, however, if 3,4-epoxidation of phenacetin (Pathway 2, Fig. 2B), were the only mechanism of activation of phenacetin. Finally, no ^{18}O would be retained in the mercapturic acid if it were formed solely by N-hydroxylation of phenacetin followed by sulfation or glucuronidation. The finding that approximately 90% of the ^{18}O was retained thus suggests that most of the reactive metabolite arises by oxidative deethylation of phenacetin to acetaminophen, followed by further metabolism of acetaminophen. The remaining fraction of reactive metabolite might come from several pathways including decomposition of an N-hydroxylated glucuronide or sulfate conjugate of phenacetin (Fig. 2C) or

possibly epoxidation pathway as shown in Fig. 2B.

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