Reactive Metabolites of Phenacetin and Acetaminophen: A Review
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Phenacetin can be metabolized to reactive metabolites by a variety of mechanisms. (1) Phenacetin can be N-hydroxylated, and the resulting N-hydroxyphenacetin can be sulfated or glucuronidated. Whereas phenacetin N-O sulfate immediately rearranges to form a reactive metabolite which may covalently bind to protein, phenacetin N-O glucuronide slowly rearranges to form reactive metabolites. Incubation of the purified phenacetin N-O glucuronide under a variety of conditions suggests that N-acetyl-p-benzoquinone imine is a reactive metabolite. This metabolite covalently binds to protein, reacts with glutathione to form an acetaminophen-glutathione conjugate, is reduced by ascorbate to acetaminophen or is partially hydrolyzed to acetamide. (2) Phenacetin can be O-deethylated to acetaminophen, and acetaminophen can be converted directly to a reactive metabolite which may be also N-acetyl-p-benzoquinone imine. (3) Phenacetin can be sequentially N-hydroxylated and O-deethylated to N-hydroxyacetaminophen which spontaneously dehydrates to N-acetyl-p-benzoquinone imine. (4) Phenacetin can be 3,4-epoxidated to form an alkylating and an arylating metabolite. In the presence of glutathione, a S-ethylglutathione conjugate and an acetaminophen-glutathione conjugate are formed. In the absence of glutathione, the alkylating metabolite may bind to protein and the arylating metabolite is completely hydrolyzed to acetamide and another arylating metabolite which may bind to protein. The structures of the alkylating and arylating metabolites are unknown.

Control experiments have shown that in pathway (1) the phenolic oxygen of the acetaminophen-glutathione conjugate is derived from water, whereas in pathways (2) and (3) the phenolic oxygen of this metabolite is derived from phenacetin. In pathway (4) the phenolic oxygen was 50% derived from molecular oxygen and 50% from phenacetin. Administration of [p-14C]phenacetin to hamsters revealed only a 10% loss of 14C in the acetaminophen mercapturic acid (the further metabolic product of the glutathione conjugate) which suggests that, in the hamster, pathways (2) and/or (3) are the primary mechanism of conversion of phenacetin to reactive metabolites in vivo.

Introduction
Phenacetin (p-ethoxyacetanilide) and acetaminophen (paracetamol, p-hydroxyacetanilide) are two of the more popular analgesic antipyretics. Acetaminophen was originally introduced in 1893, whereas phenacetin was introduced as an analgesic in 1897 (1). Phenacetin has been a commonly used drug for many years; however, acetaminophen has become a widely used drug only in the last couple of decades (2).

While there has been a dramatic increase in acetaminophen consumption in recent years, there has been a sharp decrease in worldwide use of phenacetin. This is primarily attributable to kidney toxicities (analgesic nephropathy) and possible tumors associated with the abuse of phenacetin (3, 4). Abuse of this drug has been defined as the consumption of at least one gram per day for at least three years (3, 4). Analgesic nephropathy is characterized by interstitial nephritis and progressive reduction in renal size, secondary to repeated episodes of papillary necrosis (3, 4). A high incidence of tumors of the urinary tract has also been correlated with abuse of this drug (5, 6).

An animal model for phenacetin-induced nephrotoxicity has not been developed; however, in rats (7) and hamsters (8) acute doses have been shown to produce a centrilobular hepatic necrosis. Chronic administration of phenacetin to rats has been recently reported to produce tumors of the urinary tract (9). The mechanism of these toxicities is unclear.

Acetaminophen, which is the primary phenacetin metabolite, has not been shown to be carcinogenic, but in acute doses it produces a centrilobular hepatic necrosis in man and experimental animal (7, 10). The toxicity is mediated by a reactive metabolite which covalently binds to protein and is formed by the cytochrome P-450 mixed function oxidase system (11-14) (Fig. 1). Studies with analogs of acetaminophen have suggested that N-oxidation may be
an important step in conversion of acetaminophen to the reactive metabolites (15-17). For example, it was shown that the enzyme that N-hydroxylates p-chloroacetanilide also may convert acetaminophen to a reactive metabolite. Since Calder et al. (18) showed that N-acetyl-p-benzoquinone imine was electrophilic, it was postulated that acetaminophen was N-hydroxylated and then immediately dehydrated to produce the reactive metabolite N-acetyl-p-benzoquinone imine (19).

In this work we report mechanisms of formation of reactive metabolites of phenacetin which may be important in its toxicities, and in particular, the mechanism of phenacetin-induced hepatic necrosis in hamsters. Previous work on this hepatic toxicity had suggested that this necrosis may be mediated by a reactive metabolite which binds to protein (8).

Materials and Methods

Chemicals

N-Hydroxyphenacetin was synthesized by zinc reduction of p-nitrophenetole followed by acetylation with acetyl chloride (17). Nitrosation of [15O] phenol, followed by reduction and acetylation, yielded [p-15O] acetaminophen. Treatment of the [p-15O]acetaminophen with ethyl iodide yielded [p-O]phenacetin (20). N-Hydroxyacetaminophen was a gift of Dr. Ian Calder, University of Melbourne, Australia.

Assays

Metabolites were isolated, purified and quantified by either high pressure liquid chromatography or thin-layer chromatography. Microsomal incubation mixtures were performed by using hamster liver microsomes. Sulfation assays were conducted with 100,000 g rat liver supernatant which had been chromatographed on Sephadex G-25 to remove glutathione (21). Glucuronidation assays were performed by using Triton X-100-treated rat liver microsomes (21).

Results and Discussion

N-Hydroxylation of Phenacetin

Previous work suggested that phenacetin-induced hepatotoxicity in hamsters was mediated by a reactive metabolite which covalently bound to protein (8). Since N-oxidation of acetaminophen was postulated to be an important step in acetaminophen-induced hepatotoxicity (11-15, 19), the possibility was examined that phenacetin was N-hydroxylated to form a reactive metabolite. N-Hydroxyphenacetin was synthesized, and a chromatographic assay was developed to separate N-hydroxyphenacetin from other known phenacetin metabolites (17). A metabolite with the chromatographic characteristics of N-hydroxyphenacetin was isolated from an incubation mixture of phenacetin, hamster liver microsomes, and NADPH. Mass spectral analysis indicated that the metabolite had a molecular ion and a fragmentation pattern identical to that of synthetic N-hydroxyphenacetin (17). Comparison of the rate of phenacetin N-hydroxylation (17) to phenacetin O-deethylation (acetaminophen formation) (22) revealed that phenacetin was N-hydroxylated at approximately 10% the rate it was O-deethylated (Table 1). Whereas both activities were stimulated by pretreatment of hamsters with 3-methylcholanthrene, only the O-deethylation activity was induced by phenobarbital pretreatment.

The possibility was also investigated that N-hydroxyphenacetin may be converted to a reactive metabolite and be important in phenacetin-induced hepatotoxicity. However, when [acetyl-14C]N-hydroxyphenacetin was incubated in the presence or

| Treatment | Metabolic product, nmole/min/mg microsomal protein | N-Hydroxyphenacetin | Acetaminophen |
|-----------|-----------------------------------------------|---------------------|---------------|
| None      | 0.24                                          | 2.3                 |
| Phenobarbital | 0.25                                        | 4.5                 |
| 3-Methylcholanthrene | 0.39                                           | 5.5                 |

*Hamsters were pretreated with either phenobarbital or 3-methylcholanthrene and rates of N-hydroxylation (17) or O-deethylation determined (22).
absence of NADPH, covalent binding to protein was not detected (17).

Conversion of N-Hydroxyphenacetin to Reactive Metabolites by Sulfation and Glucuronidation

Since work in the Millers' laboratory showed that sulfation of the N-hydroxy compounds may lead to reactive metabolites (29), the role of sulfation or glucuronidation in the formation of a reactive metabolite was investigated. N-Hydroxyphenacetin was readily sulfated and glucuronidated in vitro incubation mixtures (21). Furthermore, covalent binding to protein was detected when [acetyl-14C]N-hydroxyphenacetin was used as a substrate (21, 24). Whereas the N-O sulfate of N-hydroxyphenacetin proved to be a very unstable intermediate, the phenacetin N-O glucuronide was a semistable compound which could be isolated in pure form. When the purified phenacetin N-O glucuronide was incubated in Tris buffer, pH 7.4 at 37°C, the half-life was approximately 9.0 hr, and acetaminophen, acetamide, phenacetin and 2-hydroxyphenacetin glucuronide were identified as breakdown products. Inclusion of ascorbic acid in the incubation mixture increased acetaminophen formation and decreased acetamide formation. In the presence of bovine serum albumin, covalent binding to protein was detected and acetaminophen and acetamide formation were inhibited. Glutathione inhibited covalent binding to bovine serum albumin and an acetaminophen-glutathione conjugate was formed. It was thus postulated that phenacetin N-O glucuronide decomposed to yield N-acetyl-p-benzoquinone imine. This metabolite could be reduced to acetaminophen, covalently bind to protein, react with glutathione to form an acetaminophen-glutathione conjugate or hydrolyze to acetamide, plus presumably p-benzoquinone. Neither ascorbic acid, bovine serum albumin nor glutathione altered phenacetin and 2-hydroxyphenacetin glucuronide formation. Thus, the latter does not lead to N-acetyl-p-benzoquinone imine (24).

Subsequent work suggested that multiple reactive intermediates were formed during the breakdown of phenacetin N-O glucuronide (Fig. 2) (25). When the phenacetin N-O glucuronide was allowed to decompose in the presence of sodium phosphate, pH 7.4, 3-hydroxyphenacetin phosphate was characterized as a metabolite. This product was identified through comparison of its chromatographic and mass spectral properties to synthetic standards and was apparently formed from an intermediate that led to the postulated N-acetyl-p-benzoquinone imine. Thus, increasing the phosphate buffer concentration in the incubation mixture increased 3-hydroxyphenacetin phosphate formation and decreased acetaminophen and acetamide formation (Fig. 3). Phenacetin and 2-hydroxyphenacetin glucuronide formation were not altered by phosphate suggesting their formation is unrelated to 3-hydroxyphenacetin phosphate. When phenacetin N-O glucuronide was incubated in the presence of bovine serum albumin and varying concentrations of phosphate buffer, covalent binding to protein was inhibited (Fig. 4). Kinetic analysis of these data (Figs. 3 and 4) indicated that at infinite phosphate concentration, 40% of the total breakdown products of phenacetin N-O glucuronide could be trapped as 3-hydroxyphenacetin phosphate and that acetamide formation could be completely inhibited. Acetaminophen formation, however, could be only partially inhibited (Fig. 3), because even at high phosphate concentration 11% of the decomposition products of phenacetin N-O glucuronide formed acetaminophen. In the presence of bovine serum albumin where acetaminophen formation was inhibited, 11% of the decomposition products of phenacetin N-O glucuronide which were covalently bound to protein could not be inhibited by phosphate. These data suggested that covalent binding to protein may occur by two different reactive metabolites. One reactive metabolite was trapped by phosphate, before it could release acetamide, while formation of the other reactive metabolite was not affected by phosphate. In the absence of bovine serum albumin, both reactive metabolites were converted to acetaminophen. The intermediate which reacted with phosphate was postulated to be the ni-
trenium ion of phenacetin formed by heterolytic cleavage of N-O bond of phenacetin N-O glucuronide. The phosphate may possibly react initially in the 4 position and rearrange to the stable 3-hydroxyphenacetin phosphate. In the absence of phosphate, the phenacetin nitrenium reacts with a hydroxyl ion and the intermediate which is formed decomposes to yield N-acetyl-p-benzoquinone imine [experiments revealed that the phenolic oxygen of acetaminophen and the acetaminophen-GSH conjugate were derived from H$_2$O (20)]. The structure of the reactive metabolite which is not inhibitable by phosphate is unknown. The postulated mechanisms of breakdown for phenacetin N-O glucuronide are presented in Figure 2 (25).

Evidence for N-Acetyl-p-Benzoinone Imine as the Reactive Metabolite of Acetaminophen

Although N-acetyl-p-benzoquinone imine was postulated to be the reactive metabolite of acetaminophen, no evidence was initially presented to support this hypothesis (15, 19). Thus the data on the decomposition of phenacetin N-O glucuronide were very revealing (24), because they suggested that N-acetyl-p-benzoquinone imine may be the reactive metabolite of acetaminophen which could covalently bind to protein or react with glutathione to form an acetaminophen-glutathione conjugate. Moreover, the data on the breakdown of phenacetin N-O glucuronide (24) suggested that ascorbic acid should block covalent binding to protein of the reactive metabolite of acetaminophen and that acetamide may be a metabolite. Subsequent investigations showed that, indeed, ascorbic acid could block covalent binding of the reactive metabolite of acetaminophen in microsomal incubation mixtures (2, 21, 26) (Fig. 5). Furthermore, acetamide was found to be a microsomal metabolite of acetaminophen (Fig. 6). Comparison of 14C-acetamide formation to covalent binding of [14C]-acetaminophen to protein revealed that acetamide was formed at a rate of approximately 25% the rate of covalent binding (27).

An intermediate which could lead to the formation of N-acetyl-p-benzoquinone is N-hydroxyacetaminophen. Indeed, initial work with the acetaminophen analogs p-chloroacetanilide (15, 16) and phenacetin (17) showed that these analogs were N-hydroxylated, apparently by the same cytochrome P-450 species which converted acetaminophen to a reactive metabolite. These data supported the concept that acetaminophen was initially N-hydroxylated and that once formed, the N-hydroxy derivative immediately dehydrated to the reactive metabolite N-acetyl-p-benzoquinone imine. Fur-

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**Figure 3.** Effect of phosphate buffer on the breakdown of phenacetin N-O glucuronide. Purified phenacetin N-O glucuronide was allowed to break down in the presence of 0.05M Tris-HCl buffer, pH 7.4, and in the presence of varying concentrations of sodium phosphate, pH 7.4 (25).

**Figure 4.** Effect of phosphate buffer on covalent binding to protein of reactive metabolites formed during breakdown of phenacetin N-O glucuronide. Phenacetin N-O glucuronide, 50 (Δ) or 100 (○) nmole, was incubated with 10 mg bovine serum albumin in a total volume of 1 mL of 0.05M Tris-HCl, pH 7.4, and varying amounts of sodium phosphate, pH 7.4 (25).
Figure 5. Effect of ascorbic acid on covalent binding to protein of microsomal generated reactive metabolite of acetaminophen. Radiolabeled acetaminophen was incubated with hamster liver microsomes, an NADPH-generating system and varying concentrations of ascorbic acid. Ascorbic acid at these concentrations did not significantly inhibit other cytochrome P-450 reactions such as ethyl morphine N-demethylation or 3-hydroxylation of acetaminophen (26).

Figure 6. Time course for microsomal conversion of [acetyl-14C]acetaminophen to covalently bound metabolite and acetamide. [Acetyl-14C]acetaminophen was incubated with hamster liver microsomes and an NADPH-generating system. Covalent binding was determined by a solvent extraction assay whereas acetamide was determined by an HPLC assay (2, 31).

Furthermore, when N-hydroxyacetaminophen was synthesized by Healey et al. (28) and Gemborys et al. (29), it was found to dehydrate to N-acetyl-p-benzoquinone imine as was postulated. However, the compound did not immediately dehydrate but rather had a half-life of approximately 15 min, which indicates that N-hydroxyacetaminophen may not be a quantitatively important intermediate leading to the acetaminophen reactive metabolite. Moreover, N-hydroxyacetaminophen was not detected as a microsomal metabolite of acetaminophen; however, it was detected as a microsomal metabolite of N-hydroxyphenacetin (Fig. 7) (30). Since more covalent binding was detected with acetaminophen than with N-hydroxyphenacetin, this suggested that acetaminophen is not activated to a reactive metabolite via N-hydroxyacetaminophen (29). Inasmuch as the data indicated that N-acetyl-p-benzoquinone imine was the reactive metabolite, it was postulated that acetaminophen was initially N-oxygenated at the active site of cytochrome P-450 and that this species broke down, as shown in Figure 8, to yield N-acetyl-p-benzoquinone imine instead of N-hydroxyacetaminophen (31).

Conversion of Phenacetin to Reactive Metabolites by 3,4-Epoxidation

Incubation of [ring-3H]phenacetin with hamster liver microsomes and NADPH led to covalent binding to the microsomal protein (22). Since inclusion of an excess of unlabeled acetaminophen in the incubation mixture did not decrease the amount of covalent binding, the binding is apparently not mediated by an initial deethylation to acetaminophen followed by metabolism of acetaminophen. Inclusion of glutathione in the microsomal incubation mixture blocked covalent binding to protein and a glutathione conjugate was formed. Surprisingly, when the conjugate was analyzed, it was an acetaminophen-glutathione conjugate. Since Daly (32) had presented evidence that other acetanilides, such as p-chloroacetanilide, may be metabolized by 3,4-epoxidation, the possibility was investigated that phenacetin may be analogously 3,4-epoxidated (22). If phenacetin 3,4-epoxide were formed, it may rearrange, producing N-acetyl-p-benzoquinone imine. The importance of this mechanism was investigated by incubating phenacetin with microsomes, NADPH and glutathione in the presence of a molecular oxygen-18 atmosphere. If 3,4-epoxidation were the mechanism of activation of phenacetin, 15O may be expected to be quantitatively incorporated into the phenolic oxygen of the acetaminophen-glutathione conjugate. Mass spectral analysis of the acetaminophen-
glutathione conjugate from this microsomal incubation mixture (Table 2) revealed that there was only a 50% incorporation of molecular oxygen into the metabolite (22). The origin of the remaining 50% of the phenolic oxygen in the acetaminophen-glutathione conjugate was investigated by synthesizing \([p-^{18}O]\)phenacetin and using it as a substrate in an incubation mixture containing hamster liver microsomes, NADPH, and glutathione in the presence of an air atmosphere (20). As shown in Table 3, the \([p-^{18}O]\)phenacetin used in this experiment contained 48% \(^{18}O\), whereas the acetaminophen-glutathione conjugate isolated from the microsomal incubation mixture contained 23% \(^{18}O\). Thus there was approximately a 50% loss of \(^{18}O\) from phenacetin during the activation of phenacetin to a reactive metabolite and this loss corresponded to a 50% incorporation of molecular oxygen into the metabolite. These findings suggest that phenacetin is converted to a reactive metabolite by an initial 3,4-epoxidation and that then an intermediate is formed in which the oxygen derived from molecular oxygen and the oxygen from phenacetin are equivalent (20).

Further insight into the mechanism of formation of phenacetin reactive metabolites formed by 3,4-epoxidation was obtained by studying the microsomal metabolism of [ethyl-\(^{14}C\)]phenacetin, [acetyl-\(^{14}C\)]phenacetin and [ring-\(^{14}C\)]phenacetin. Surprisingly, when [ethyl-\(^{14}C\)]phenacetin was incubated with microsomes and NADPH, covalent binding of the radiolabel to microsomal protein was observed (Table 4). When glutathione was included in the incubation mixture, protein covalent binding was inhibited and a S-ethyl glutathione conjugate was formed. Deuterium isotope experiments using [ethyl-d5]phenacetin revealed that none of the protons was lost in the formation of the S-[ethyl-d5] glutathione conjugate. The structure of the alkylating metabolite is unknown; however, its covalent binding to protein can be inhibited by ascorbate (Fig. 9). Presumably, the reactive metabolite is not the iminoquinone hemiketal of phenacetin (33) since this species would be expected to be a decomposition product of phenace-

![Figure 7](image7.png)

**Figure 7.** Time course of \(N\)-hydroxyacetaminophen formation and covalent binding during the microsomal metabolism of acetaminophen and \(N\)-hydroxyphenacetin. Radiolabeled acetaminophen or radiolabeled \(N\)-hydroxyphenacetin was incubated with hamster liver microsomes and NADPH. At the indicated time either covalent binding to protein or \(N\)-hydroxyacetaminophen formation was measured by HPLC (30).

![Figure 8](image8.png)

**Figure 8.** Postulated mechanism for metabolism of acetaminophen to \(N\)-acetyl-p-benzoquinone imine.

![Diagram](image9.png)

**Table 2. Role of molecular \(^{18}O\) in the microsomal metabolism of phenacetin derivatives.**

| Substrate       | Metabolite                                | \(^{18}O\), % |
|-----------------|-------------------------------------------|--------------|
| Acetaminophen   | Acetaminophen-GSH conjugate               | 0            |
| Phenacetin      | Acetaminophen-GSH conjugate               | 40           |
| Acetaminophen   | Acetaminophen                             | 0            |
| Acetanilide     | Acetaminophen                             | 80           |

\(^{18}O\) Acetaminophen, phenacetin, and acetanilide were incubated in three separate microsomal incubation mixtures under the same \(^{18}O\) atmosphere (80% \(^{18}O\)). The acetaminophen-glutathione conjugates were isolated from the acetaminophen and phenacetin incubation mixtures. Also, acetaminophen was isolated from the phenacetin and acetanilide incubation mixtures. The \(^{18}O\) content of each metabolite was determined by mass spectrometry (22).

![Table 3](image10.png)

**Table 3. \(^{18}O\) content of acetaminophen-GSH conjugates from \(^{18}O\)-acetaminophen and \(^{18}O\)-phenacetin microsomal incubation mixtures.**

| Substrate       | Metabolite                                | \(^{18}O\), % |
|-----------------|-------------------------------------------|--------------|
| Acetaminophen   | Acetaminophen-GSH conjugate               | 48           |
| Phenacetin      | Acetaminophen-GSH conjugate               | 45           |
|                 | Acetaminophen-GSH conjugate               | 48           |
| Acetaminophen   | Acetaminophen                             | 23           |
| Acetaminophen   | Acetaminophen                             | 46           |

\(^{18}O\) The \(^{18}O\) was substituted in the 4 position of both acetaminophen and phenacetin. Incubations were run in an air atmosphere, and the \(^{18}O\) content of the metabolites was determined by mass spectrometry (20).
FIGURE 9. Effect of glutathione and ascorbic acid on covalent binding to protein of various labeled phenacetins: (■) [acetyl-14C]phenacetin, (△) [ethyl-14C]phenacetin, and (●) ring-14C]phenacetin. The labeled phenacetins were incubated in separate microsomal incubation mixtures with microsomes, an NADPH regeneration system and varying concentrations of either glutathione or ascorbate. In each incubation mixture covalent binding to protein was determined. Also, with [ethyl-14C]phenacetin, a S-ethyl-GSH conjugate (△) was quantitated, whereas with [acetyl-14C]acetaminophen (□) and [ring-14C]acetaminophen (○) an acetaminophen-GSH conjugate was quantitated.

Table 4. Comparison of microsomal metabolism of various radiolabeled phenacetin and acetaminophen derivatives to reactive metabolites.

| Substrate          | Protein covalent binding, nmole/mg/10 min | Acetamide, nmole/mg/10 min |
|--------------------|------------------------------------------|----------------------------|
| [Acetyl-14C]Phenacetin | 0.04 ± 0.03                              | 2.17 ± 0.61                |
| Ring-14C]Phenacetin   | 1.65 ± 0.08                              | —                          |
| [Ethyl-14C]Phenacetin | 0.89 ± 0.06                              | —                          |
| [Acetyl-14C]Acetaminophen   | 0.83 ± 0.02                             | 0.18 ± 0.03                |
| Ring-14C]Acetaminophen    | 1.07 ± 0.05                             | —                          |

*Phenacetin metabolism and acetaminophen metabolism were determined by using hamster liver microsomes (27, 33). Amide is known to be formed from quinone imine, presumably both the acetylated reactive intermediates are quinone imine derivatives. The deacetylated reactive metabolite, however, may be p-benzoquinone. This species is an electrophile and has been previously reported to conjugate with cysteine.

Mechanism of Conversion of Phenacetin to Reactive Metabolites In Vivo

The above data suggest that phenacetin may be metabolized to reactive metabolites by at least four different pathways: (1) N-hydroxylation followed by sulfation or glucuronidation (17, 21, 29); (2) deethylation to acetaminophen followed by oxidation of acetaminophen (2, 12, 13, 30); (3) N-hydroxylation followed by O-deethylation (17, 27-29); and (4) 3,4-epoxidation (20, 22, 32). Each of these mechanisms yields an arylating metabolite which reacts with glutathione to produce an acetaminophen-glutathione conjugate. In vivo, this conjugate would be further metabolized and excreted in the urine as an acetaminophen-N-acetylcysteine conjugate (acetaminophen mercapturic acid (2)). The importance of these various pathways in the in vivo metabolism of phenacetin in hamster was investigated by studying the metabolism of [p-3H]phenacetin to the urinary acetaminophen merapturic acid (20). If pathways (2) or (3) were the major mechanisms of conversion of phenacetin to reactive metabolites in vivo, no loss of 3H would occur in the acetaminophen mercapturic acid. If pathway (4) were the major mechanism of activation of phenacetin in vivo, there would be a 50% loss of 3H in the acetaminophen mercapturic
acid. If pathway (1) were the major mechanism of activation of phenacetin to reactive metabolite(s) \textit{in vivo}, there would be a 100% loss of $^{18}$O in the acetaminophen mercapturic acid. Two hamsters were thus administered [$p^{-18}$O]phenacetin (50 mg/kg) and the urinary acetaminophen mercapturic acid isolated. This metabolite represented approximately 15% of the total administered dose. Mass spectral analysis of this metabolite (Table 5) revealed approximately a 10% loss of $^{18}$O in the acetaminophen mercapturic acid. The data indicate that \textit{in vivo} reactive metabolites of phenacetin are formed principally by mechanisms which do not lead to loss of the para oxygen of phenacetin such as a deethylation to acetaminophen and subsequent activation of acetaminophen (pathway 2) or $N$-hydroxylation of phen-
aminophen followed by activation of acetaminophen and/or by N-hydroxylation of phenacetin followed by O-deethylation to N-hydroxyacetaminophen which spontaneously dehydrates. Since both acetaminophen and N-hydroxyacetaminophen produce hepatic necrosis, the hepatic toxicity observed with large doses of phenacetin in hamsters may be mediated by either mechanism or by a combination of the two.

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