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The Formation of Arylating and Alkylating Metabolites of Phenacetin in Hamsters and Hamster Liver Microsomes

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SUMMARY

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Phenacetin (4′-ethoxyacetanilide) is metabolized by multiple pathways, some of which can lead to the formation of reactive electrophilic products that may combine with glutathione or tissue macromolecules. Hamster liver microsomes converted ring-labeled [^{14}C]phenacetin to an arylating metabolite that irreversibly bound to microsomal protein (1.65 ± 0.08 nmoles/mg/10 min). The binding was decreased more than 80% in the presence of 2.5 mM ascorbic acid or 1.0 mM glutathione. Under the same conditions, radiolabel from [*ethyl*-^{14}C]phenacetin bound to microsomal protein only to about one-half the extent of the ring label (0.89 ± 0.06 nmol/mg/10 min), although binding was decreased to the same levels as ring-labeled phenacetin by either ascorbate or glutathione. In striking contrast, radiolabel from [*acetyl*-^{14}C]phenacetin did not bind to microsomal protein, but rather yielded significant amounts of acetamide and acetic acid. However, both acetyl- and ring-labeled phenacetin formed approximately equivalent amounts of a glutathione conjugate of acetaminophen in the presence of hamster liver microsomes and glutathione, whereas ethyl-labeled phenacetin formed lesser amounts of *S*-ethyl glutathione. Metabolites found in hamster urine included 3-cysteinylacetaminophen and acetaminophen-3-mercapturate which maintained radiolabel from both ring- and acetyl-labeled phenacetins, and *S*-ethylcysteine and *S*-ethyl-*N*-acetylcysteine which maintained radiolabel from ethyl-labeled phenacetin. Deuterium substitution for hydrogen in the ethyl group of phenacetin showed that no deuterium was lost in the ethyl conjugates derived from phenacetin either *in vitro* or *in vivo*. These results show that phenacetin can be metabolized to both reactive arylating and alkylating agents by hamsters and hamster liver microsomes, and indicate that the metabolites involved in sulfur-ether conjugate formation are different from those that bind to tissue macromolecules.

INTRODUCTION

High doses of phenacetin, a mild analgesic of the acetanilide group, have been implicated in renal injury in man (1) and cause hepatic necrosis in hamsters (2, 3). Previous studies have revealed that liver preparations from hamsters convert phenacetin to chemically reactive metabolites along at least three different pathways (3–9). In one pathway, phenacetin can undergo oxidative *O*-deethylation to form acetaminophen, which is converted by further oxidation to a reactive metabolite (3). In a second pathway, phenacetin can be converted to an arylating metabolite, presumably via an arene oxide, since oxygen from molecular oxygen is incorporated into the phenolic group of an acetaminophen-glutathione conjugate that arises from a reactive phenacetin metabolite (4). In the third pathway, phenacetin is oxidized to *N*-hydroxyphenactin (5), which can then be converted to *N*-*O*-sulfate and *N*-*O*-glucuronide conjugates that decompose spontaneously to reactive metabolites (6, 7).

These three pathways have been examined further in hamsters *in vivo* using phenacetin specifically labeled with stable isotopes (3, 8, 9). The results of these studies have indicated that all three pathways are probably involved in reactive metabolite formation *in vivo*, and

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that under the conditions of the experiments, dealkylation of phenacetin to acetaminophen and further metabolism of the acetaminophen that is formed is the major pathway for reactive metabolite formation.

The present study was undertaken to determine the fate of three separate functional moieties of the phenacetin structure with regard to reactive metabolite formation. Experiments with phenacetin specifically labeled with ^{14}C in the aromatic ring, acetyl group, and ethyl group revealed that mechanisms other than those that already have been described for glutathione conjugate formation are involved in protein adduct formation. In addition, labeling the ethyl group with deuterium has allowed us to determine that this moiety can act as an alkylating agent during the metabolism of phenacetin in hamsters.

MATERIALS AND METHODS

Glutathione, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Company, St. Louis, Mo., and thin-layer chromatographic plates were obtained from either Matheson, Coleman, and Bell, Norwood, Ohio, or Waters Associates, Milford, Mass., and were of nanograde purity. All other chemicals were of the purest grade commercially available. Male golden Syrian hamsters (60–80 g) were obtained either from the Animal Production Unit at the National Institutes of Health or from Simonson Laboratories, Gilroy, Calif.

Syntheses of labeled phenacetins. Ring-labeled [^{14}C]phenacetin was prepared from [2,6-^{14}C]-4-nitrophenol (ICN Radiochemicals, Irvine, Calif., by the following procedure. [2,6-^{14}C]-*p*-Nitrophenol (250 μCi, 20 mCi/mmole) was combined with *p*-nitrophenol (35 mg) in acetone (1.0 ml) in a 3-ml Reacti-Vial (Pierce Chemical Company, Rockford, Ill.) containing anhydrous potassium carbonate (40 mg). To the rapidly stirred slurry was added ethyl bromide (50 μl). The Reacti-Vial was sealed with a silicone-Teflon disc and heated at reflux for 2 hr, after which an additional 50 μl of ethyl bromide were added through the disc. After stirring for an additional 2 hr at reflux, the reaction mixture was cooled and the acetone was evaporated under a gentle stream of nitrogen. The remaining reaction solid was partitioned between diethyl ether and distilled water. The ether extract was washed with 1 N NaOH solution followed by two water washes and dried over anhydrous magnesium sulfate overnight. The drying agent was removed by filtration and ether was removed under a gentle stream of nitrogen. Without further purification the yellow solid *p*-nitrophenetole was reductively acetylated by hydrogenation in ethanol (5 ml) over 10% Pd/C (10 mg) and excess acetic anhydride (0.5 ml). The final product, [2′,6′-^{14}C]-4′-ethoxyacetanilide, or ring-labeled phenacetin, was purified as described for *p*-nitrophenetole after filtration of the reaction mixture through Celite filter-aid to remove the catalyst, giving an over-all yield of 63% after recrystallization from methanol-water (specific activity 0.92 mCi/mmole).

[*ethyl*-^{14}C]Phenacetin was synthesized in a similar manner, using *p*-nitrophenol (Eastman Organic Chemicals, Rochester, N. Y.) and [1-^{14}C]ethyl bromide (New England Nuclear Corporation, Boston, Mass.) to give 4′-[1-^{14}C-*ethoxy*]acetanilide or ethyl-labeled phenacetin, in 58% over-all yield, specific activity 1.24 mCi/nmole. 4′-[1,1,2,2,2-$^{2}H_5$-*ethoxy*]Acetanilide, or d_5-phenacetin, was prepared by the same procedure using [1,1,2,2,2-$^{2}H_5$]bromethane (Merck and Company/Isotopes, St. Louis, Mo.).

[*acetyl*-^{14}C]Phenacetin was prepared from *p*-phenetidine (Eastman Organic Chemicals, Rochester, N. Y.) and [1-^{14}C]acetyl chloride (New England Nuclear Corporation) via direct acetylation in ethyl acetate, in an over-all yield of 85%, specific activity 5.40 mCi/mmole.

Prior to use, all radiolabeled compounds were further purified by thin-layer chromatography on 1000-μm silica gel GF plates (20 × 20 cm) developed in ethyl ether; R_F of phenacetin, 0.5–0.6.

Synthesis of S-ethylglutathione. *S*-Ethylglutathione was prepared by the procedure of Vince *et al.* (10) in 67% over-all yield.

Metabolism and irreversible binding experiments. Microsomal incubations involving irreversible binding determinations and trapping of reactive metabolites with glutathione were carried out as previously described (4, 8, 9). For *in vivo* studies, hamsters were administered the appropriately labeled phenacetin analogue (50 mg/kg, i.p., containing approximately 20 μCi of radiolabel) dissolved in 0.15 ml of Tween 80, and each animal was kept in a separate all-glass metabolism cage. Urine was collected for 24 hr over Dry Ice and stored at −20° until work-up.

Metabolite isolation and analysis. Glutathione conjugates of phenacetin were isolated by a combination of HPLC[3] and thin-layer chromatography. Microsomal protein was precipitated from 3-ml incubations, after 10-min reaction periods at 37°, by the addition of 3 ml of ice-cold methanol. The supernatant remaining after centrifugation of protein at 1000 × *g* for 20 min was filtered through a 0.5-μm Millipore filter. A 0.5-ml aliquot of this supernatant was evaporated under a gentle stream of nitrogen and reconstituted in 100 μl of glass-distilled water. Samples of this solution (usually 50 μl) were then analyzed by high-pressure liquid chromatography as previously described (11), using a slightly modified mobile phase consisting of 10% methanol-1% acetic acid-89% water. The major ultraviolet-visible glutathione conjugate eluting at 12–13 min was found to be the same 3-(*S*-glutathionyl)-acetaminophen conjugate that is formed from acetaminophen under similar incubation conditions, as determined by methods previously outlined (11). This conjugate retained the ^{14}C label from both acetyl- and ring-labeled phenacetin, but not that from ethyl-labeled phenacetin. Using ethyl-labeled phenacetin, another glutathione conjugate not detectable at 254 nm but containing radioactivity, appeared in the HPLC void volume. This fraction was collected and rechromatographed on a 500-μm Avicel F plate which was then developed with 60% 1-butanol-15% acetic acid-25% water. The radiolabel co-chromatographed with *S*-ethylglutathione (R_F 0.43) and could be recrystallized to constant specific activity

[3] The abbreviations used are: HPLC, high-pressure liquid chromatography; TMS, trimethylsilyl.

with synthetic *S*-ethylglutathione using methanol-water mixtures.

Acetic acid and acetamide were analyzed by the addition of 1 μl of acetic acid and 500 μg of acetamide as unlabeled carriers to supernatants obtained after microsomal incubations containing acetyl-labeled phenacetin as the substrate. Aliquots (1 ml) of the supernatants were then slowly passed through a small column containing an 8-ml bed volume of XAD-2 resin. This procedure removed most of the radioactive materials other than acetic acid and acetamide. Both compounds could then be analyzed by collecting in tubes immersed in Dry Ice, the peaks corresponding to each compound as they eluted from a 5% FFAP column (1.8 m × 4 mm inner diameter) on a Varian Model 920 preparative gas chromatograph (helium flow rate 40 ml/min) with a thermal detector set at 150 mA. Using an initial column temperature of 60° for 4 min followed by approximate temperature programming at 10°/min up to 120°, the corresponding retention times of acetic acid and acetamide were 3.2 min and 9.0 min. Combined collections from five 20-μl injections were added to 10 ml of ACS scintillation cocktail (Amersham Radiochemicals, Arlington Heights, Ill). and radioactivity determinations were then made on a Beckman LS-230 liquid scintillation counter.

Radiolabeled cysteine and mercapturic acid conjugates that were formed by hamsters after doses of the variously labeled phenacetins were isolated from 24-hr urine samples by diluting aliquots with 10 volumes of distilled water, filtration of the diluted sample through a 0.5-μm Millipore filter, and collecting HPLC purified fractions from 50-μl sample injections. The cysteine and mercapturic acid conjugates of acetaminophen were identified as previously described (11) and retained ^{14}C from both acetyl- and ring-labeled phenacetins. From ethyl-labeled phenacetin additional radioactivity appeared near the void volume. This material was collected and applied to 500-μm Avicel F thin-layer plates which were developed as described for the *S*-ethylglutathione. Areas of radioactivity appeared in the regions corresponding to the R_F values for synthetic *S*-ethylcysteine (0.51) and *S*-ethyl-*N*-acetylcysteine (0.85). Samples were eluted from the plate with water and lyophilized. The samples were then derivatized with BSTFA (150° for 15 min) and analyzed by gas chromatography-mass spectrometry on a VG MicroMass 16F mass spectrometer coupled to a Varian Aerograph Model 1400 using the following conditions: column, 1.8 m × 2 mm inner diameter silanized glass packed with 3% OV-17 on Gas-Chrom Q 100/120 mesh (Applied Science, State College, Pa.): column temperature, programmed from 90°–200° at 3°/min; injector temperature, 150°; transfer line and glass jet separator temperature, 225°; ion source temperature, 250°; accelerating voltage, 4 KV; electron energy, 70 eV; ionizing current, 100 μA; ion source pressure, 2×10^{-7} Torr. Under these conditions *S*-ethylcysteine eluted as the di-TMS derivative at 6 min, based on a total ion current tracing, and gave the resulting mass spectral ions: parent ion, M/Z 293 and major fragment ions at M/Z 278, 250, 218, 176, 147, 128, 104, 75, 73, and 61. The corresponding mercapturate eluted as the mono-TMS derivative at 13 min. The mass spectrum of this metabolite is discussed in more detail under Results, along with the spectrum of the mercapturate obtained from hamster urine after the administration of the d_5-phenacetin analog.

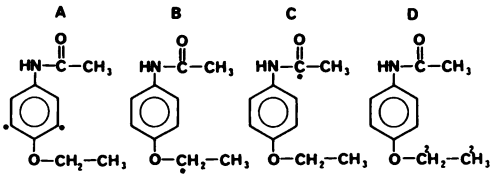


FIG. 1. *Specifically labeled phenacetin analogs*

A, [2′,6′-^{14}C]-4-′-Ethoxyacetanilide or ring-labeled phenacetin; B, 4′-[1-^{14}C-*ethoxy*]Acetanilide or ethyl-labeled phenacetin; C, 4′-Ethoxy-[1-^{14}C]acetanilide or acetyl-labeled phenacetin; D, 4′-[1,1,2,2,2-$^{2}H_5$-*ethoxy*]Acetanilide or d_5-phenacetin. The *asterisks* indicate the position of the ^{14}C label.

RESULTS

Studies in vitro. Using the three specifically radiolabeled analogs of phenacetin shown in Fig. 1, we found that the aromatic ring and ethyl group of phenacetin became irreversibly bound to microsomal protein in an NADPH-dependent reaction, whereas the acetyl group did not bind (Fig. 2). Binding levels of the ethyl group (0.89 ± 0.06 nmole/mg/10 min) were approximately 50% those of the aromatic ring (1.65 ± 0.08 nmoles/mg/10 min) when incubations were carried out in the absence of either ascorbic acid or glutathione. Both ascorbic acid and glutathione decreased the binding levels substantially; however, increasing concentrations of either compound failed to decrease the binding of the aromatic ring or ethyl label beyond a level of approximately 0.25 nmole/mg/10 min (Fig. 2).

Glutathione conjugate formation, on the other hand,

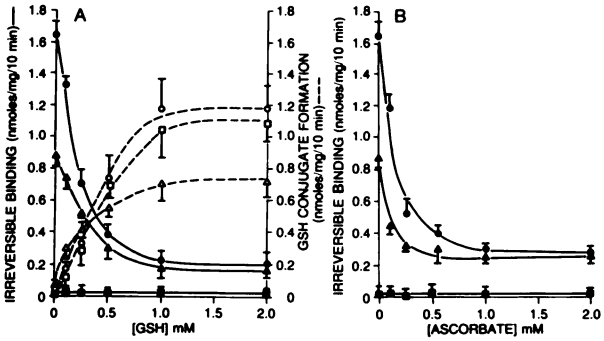


FIG. 2. *Effect of glutathione and ascorbate on the irreversible binding of labeled phenacetins to hamster liver microsomal protein*

A. Irreversible binding of ring-labeled phenacetin (●), ethyl-labeled phenacetin (▲), and acetyl-labeled phenacetin (■) to microsomal protein was determined as described under Materials and Methods from incubation mixtures (3 ml) containing hamster liver microsomes (6 mg), ^{14}C-labeled phenacetin (3 μmoles), an NADPH-generating system, and an air atmosphere. Each mixture was incubated in duplicate with varying concentrations of glutathione for 10 min at 37° in a shaker-incubator. Incubations lacking NADPH served as controls and gave binding levels of 0.05 ± 0.03 nmole/mg/10 min. Values represent the means ± standard error of the mean from four separate experiments. Glutathione conjugate formation (○), ethyl-labeled phenacetin (△), and acetyl-labeled phenacetin (□) were determined in the incubation supernatants after protein precipitation as described under Materials and Methods.

B. Irreversible binding of labeled phenacetins under conditions of increasing ascorbate concentrations. (Legends and procedures are the same as described in A.)

was found to occur with each of the labeled phenacetin analogs, including acetyl-labeled phenacetin (Fig. 2), and the formation of all glutathione conjugates was completely blocked by the addition of ascorbic acid (1 mM). The major conjugate formed from either acetyl- or ring-labeled phenacetin was identified as 3-(*S*-glutathionyl)-acetaminophen, the same glutathione conjugate that is formed from acetaminophen. A separate glutathione conjugate was formed from ethyl-labeled phenacetin, which co-chromatographed with *S*-ethylglutathione. Recrystallization to constant specific activity with synthetic *S*-ethylglutathione indicated that 70%–75% of the radioactivity present in the sample was associated with *S*-ethylglutathione. These results must be interpreted with some degree of caution since the recrystallization of these peptides really involves precipitation rather than actual crystal growth for the production of the resultant fine white powder that is analyzed. Other evidence has been presented previously (9) showing that the entire ethyl group of d_5-phenacetin does alkylate the thiol moiety of *N*-acetylcysteine when incubated with hamster liver microsomes.

Two possible pathways for loss of the acetyl group were explored using acetyl-labeled phenacetin. Acetamide was formed as a metabolite of phenacetin in hamster liver microsomes in amounts ranging from 1.5 to 2.5 nmoles/mg of microsomal protein per 10-min incubation. Acetamide formation was undetectable after the addition of ascorbic acid at 1 mM concentrations, the sensitivity of our assay, approaching formation rates of 0.1 nmole/mg/10 min. In contrast, the formation of acetic acid as a metabolite of phenacetin formed by hamster liver microsomes was not significantly inhibited by ascorbate and ranged from 4 to 6 nmoles/mg of microsomal protein per 10-min incubation. This value is close to that observed for the formation of phenetidine, the secondary metabolite of phenacetin that would arise from the hydrolysis of the amide bond of phenacetin by hamster liver microsomal amidases (unpublished results).

Studies in vivo. Radioactivity from the various ^{14}C-labeled phenacetins became irreversibly bound to hamster liver, kidney, and muscle proteins to varying extents, and the ratio of binding levels for the different labels was different in each tissue (Table 1). Because the radiolabel in both the acetyl and ethyl groups of phenacetin can be incorporated into tissue protein via acetate formation, as indicated by the expiration of carbon dioxide from the hamsters, groups of animals were pretreated with a dose of cycloheximide that is known to inhibit protein synthesis (13). This treatment significantly decreased the binding levels of radiolabel from both acetyl- and ethyl-labeled phenacetin. The largest decrease was seen with the ethyl-labeled analogue, where a significant inhibition of carbon dioxide expiration was also observed with cycloheximide pretreatment.

Hamsters administered either ring- or acetyl-labeled phenacetin excreted just over 10% of the dose as the mercapturic acid conjugate of acetaminophen, and approximately 2% of the dose appeared in the urine as the cysteine conjugate (Table 2). Hamsters administered an equivalent dose of ethyl-labeled phenacetin excreted approximately 3% of the dose as *S*-ethyl-*N*-acetylcysteine, and approximately 1% of the dose appeared in the urine as *S*-ethylcysteine (Table 2). One male hamster was administered d_5-phenacetin along with a tracer dose of ^{14}C-ethyl-labeled phenacetin, and the resulting ethyl-labeled *N*-acetylcysteine conjugate was isolated and analyzed by gas chromatography-mass spectrometry as its TMS derivative. The mass spectrum of the metabolite showed a parent ion and certain fragment ions that had shifted to higher masses by 5 a.m.u. as compared with spectra of the metabolite isolated after the administration of undeuterated phenacetin (Fig. 3). The parent ion at M/Z 263 that was observed for the TMS derivative of the unlabeled metabolite had shifted to M/Z 268. All of the corresponding ions for losses of the elements of a methyl group (M/Z 248), acetamide (M/Z 204), trimethylsilynol (M/Z 174), and carboxy-TMS (M/Z 146) shifted to higher mass by 5 a.m.u. as well, indicating retention of the d_5-ethyl group. Loss of the ethyl group apparently occurred in some fragment ions, such as that of M/Z 188, indicating loss of the elements of ethyl

TABLE 1

Irreversible binding of radiolabeled phenacetins to hamster tissues in vivo and carbon dioxide expired

Hamsters (four per group) were administered the specifically radiolabeled phenacetin as indicated (50 mg/kg, i.p.), and samples of liver, kidney, and sartorius muscle were removed 6 hr later for the determination of radiolabel irreversibly bound to the tissue protein as previously described for acetaminophen (12). Carbon dioxide was monitored as the radioactivity trapped in 200 ml of 2:1 methoxyethanol-ethanolamine contained in all-glass gas-washing bottles through which the expired air from the metabolism cages was passed at a rate of 300 ml/min. A second group of hamsters was treated with cycloheximide (2 mg/kg, i.p.) 2 hr prior to the administration of radiolabeled phenacetin. Results are expressed as means ± standard error of the mean for the four animals in each group.

Administered drug	Irreversible binding			Expired CO_2 (6 hr)
	Liver	Kidney	Muscle	
				% of dose
Ring-labeled phenacetin	2.38 ± 0.27	0.44 ± 0.08	0.03 ± 0.02	0
+ cycloheximide	2.22 ± 0.28	0.37 ± 0.06	0.02 ± 0.01	0
Acetyl-labeled phenacetin	1.94 ± 0.21	0.45 ± 0.07	0.14 ± 0.03	16 ± 3.2
+ cycloheximide	1.36 ± 0.16	0.22 ± 0.04	0.22 ± 0.02	12 ± 2.4
Ethyl-labeled phenacetin	1.95 ± 0.18	2.01 ± 0.34	0.24 ± 0.09	76 ± 5.1
+ cycloheximide	0.56 ± 0.10	0.47 ± 0.10	0.02 ± 0.01	48 ± 3.9

TABLE 2

Percentage of dose of phenacetin excreted in 24-hr hamster urine as cysteine and mercapturic acid derivatives

Male golden Syrian hamsters were administered the various radiolabeled analogues of phenacetin (50 mg/kg, i.p.) and placed in all-glass metabolic cages. Urine samples were collected for 24 hr over Dry Ice and analyzed for cysteine and mercapturic acid metabolites as described under Materials and Methods.

Administered drug	Urinary metabolite	Phenacetin excreted[a]
		% of dose
Ring-labeled phenacetin	Acetaminophen-3-mercapturate	11.1 ± 1.05
	3-(*S*-cysteinyl)-acetaminophen	2.0 ± 0.28
Acetyl-labeled phenacetin	Acetaminophen-3-mercapturate	13.4 ± 1.22
	3-(*S*-cysteinyl)-acetaminophen	2.2 ± 0.45
Ethyl-labeled phenacetin	*S*-Ethyl-*N*-acetylcysteine	3.1 ± 0.75
	S-Ethylcysteine	1.0 ± 0.51

[a] Expressed as means ± standard error of the mean for the four hamsters in each group.

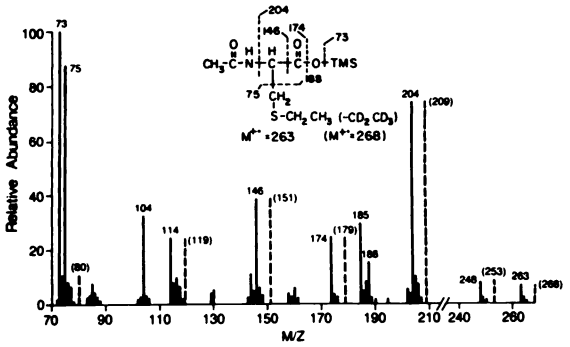


FIG. 3. *Mass spectrum of silylated S-ethyl-N-acetylcysteine metabolite isolated from hamster urine*

S-Ethyl-*N*-acetylcysteine was isolated from hamster urine as described under Materials and Methods and analyzed as its silylated derivative by gas chromatography-mass spectrometry. The fragmentation pattern is described under Results. - - -, Ions that were shifted in the mass spectrum of the same metabolite that was isolated from hamster urine after the administration of d_5-phenacetin.

methylene sulfenium radical. An ion corresponding to this fragmentation apparently contributes to the ion intensity a M/Z 75, since with the d_5 metabolite a new fragment ion appears at M/Z 80.

DISCUSSION

The results of the present studies on the irreversible binding of phenacetin to hamster liver microsomes are consistent with the results from our previous investigations which indicated that multiple pathways are involved in the metabolism of phenacetin to reactive metabolites and that the pathways leading to reactive metabolite formation from phenacetin are different from those leading to reactive metabolite from acetaminophen (4, 8, 9). The most striking difference between the binding of phenacetin and acetaminophen *in vitro* is the lack of binding of the acetyl group of phenacetin as compared with extensive binding of the acetyl group of acetaminophen (Table 3). Correlated with this result is extensive formation of acetamide from phenacetin as compared with the formation of acetamide from acetaminophen (Table 3). In addition, results from these studies show that the labeled ethyl group of phenacetin is irreversibly bound to hamster liver microsomes to about one-half the extent to which the labeled aromatic ring is bound (Table 3).

The irreversible binding of the labeled acetyl and aryl groups to microsomal protein was markedly decreased by the addition of glutathione to the incubation medium (Fig. 2A). Both acetaminophen and phenacetin form 3-(*S*-glutathionyl)-acetaminophen in the microsomal incubations to which glutathione has been added. Although this conjugate retains both the labeled acetyl and aryl group from each substrate, the two conjugates must arise from different intermediates, since the phenolic oxygen is totally retained from acetaminophen whereas only 50% of it is retained from phenacetin the remaining 50% being incorporated from molecular oxygen (4, 8). In addition, *S*-ethylglutathione is formed from the ethyl group of phenacetin.

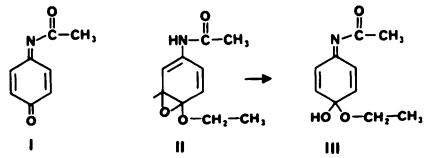
TABLE 3

Comparison of results obtained in hamster liver microsomes that relate to reactive metabolite formation from phenacetin and acetaminophen

Experiments were conducted as described in Fig. 2. Results using phenacetin as the substrate were obtained in this study, and those using acetaminophen as the substrate from ref. 14, and are expressed as means ± standard error of the mean.

Parameter	Phenacetin	Acetaminophen
	nmoles/mg/10 min	*nmoles/mg/15 min*
Irreversible binding		
^{14}C-Acetyl label	0.04 ± 0.03	1.24 ± 0.03
^{14}C-Ring label	1.65 ± 0.08	1.61 ± 0.07
^{14}C-Ethyl label	0.89 ± 0.06	
Acetamide Formation	2.17 ± 0.61	0.27 ± 0.04

Since the binding of both acetaminophen and phenacetin to microsomal protein is substantially decreased by glutathione with concomitant formation of similar thioether conjugates, the formation of similar, yet distinct, reactive intermediates is suggested. On the basis of our previous work (4, 8) and the results reported here, we postulate that acetaminophen is oxidized to *N*-acetyliminoquinone (I) and that phenacetin is oxidized to the highly activated oxirane hemiketal (II) which subsequently rearranges to a more stable iminoquinone hemiketal (III). Both I and II are quinone-like and therefore susceptible to either nucleophilic addition reactions or reduction as indicated by ascorbic acid-inhibitable binding (Fig. 2B). However, the hemiketal (III) may be more susceptible to imine hydrolysis or addition-elimination reactions at the aromatic imine carbon atom than the quinone (I) because of steric crowding around the sp^3-hybridized aromatic hemiketal carbon atom. This could account for the lack of irreversible binding of the acetyl group to microsomal protein and the substantial formation of acetamide that is observed with phenacetin as the substrate as compared with acetaminophen as the substrate. Furthermore, hydrolysis of the hemiketal generated from phenacetin could give rise to the ethylating species.



STRUCTURE 1

Results from the experiments carried out *in vivo* in the hamster are also consistent with results from our previous studies (3, 8, 9). A significant fraction of a dose of phenacetin is converted by the hamster to acetaminophen, which then can be further transformed to metabolites that become irreversibly bound to liver protein. This could account for extensive binding of the acetyl group of phenacetin *in vivo* relative to what is observed *in vitro*. In addition, hydrolysis of the amide bond of phenacetin produces acetic acid that can be incorporated into proteins. Much of the ethyl label can also be converted to acetate through the product of oxidative *O*-

deethylation, acetaldehyde. This is indicated by a substantial decrease in binding of the ethyl group and decreased expiration of carbon dioxide following cycloheximide pretreatment of the hamsters administered ethyl-labeled phenacetin (Table 2). However, the data must be viewed with considerable caution since cycloheximide has multiple effects on enzymes, including its ability to act as an inducer of certain components of the microsomal oxygenase system (15).

Because the ethyl group of phenacetin can be converted *in vivo* into acetate, and thereby appear as "bound" radiolabel, evidence that the ethyl group can act as an alkylating agent in the hamster *in vivo* is best documented by the urinary excretion of *S*-ethyl-*N*-acetylcysteine and of *S*-ethylcysteine (Table 2). Since deuterium is retained in the metabolites (Fig. 3), the entire ethyl group is apparently transferred and not simply trapped as acetaldehyde or acetate which is then further reduced. *S*-Ethyl-*N*-acetylcysteine has been previously detected in rat urine after doses of phenacetin (16, 17), and mechanisms for its formation have been suggested (16, 18).

Considerably greater amounts (~10 X) of thioether conjugates of acetaminophen are found in hamster urine as compared with *S*-ethyl conjugates after the administration of phenacetin. This provides additional support for the contention that, *in vivo*, the hamster converts phenacetin extensively to acetaminophen which is further metabolized to reactive arylating, but not alkylating, metabolites. *In vitro* the amounts of aryl- and alkyl-thioether conjugates are more nearly equivalent (Fig. 2A) because they apparently are formed from an intact reactive metabolite of phenacetin that contains both arylating and alkylating moieties. Although acetaminophen is formed *in vitro* from phenacetin by oxidative *O*-deethylation, it does not attain, within the time frame of the incubations, concentrations that are high enough to yield significant amounts of the arylating metabolite (4).

To elucidate more completely the mechanisms involved in the irreversible protein-binding reactions of acetaminophen and phenacetin will require a better characterization of the postulated intermediates and determination of the structures of the protein-bound adducts. This is particularly relevant to the binding *in vivo*, since acetate incorporation from both the acetyl and ethyl group of phenacetin (Structure 1) complicates the situation.

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