Biochemical Pharmacology, Vol. 27, pp. 1641–1649.

0006-2952/78/0615-1641$02.00/0

CONVERSION OF THE *N*-*O*-GLUCURONIDE AND *N*-*O*-SULFATE CONJUGATES OF *N*-HYDROXY-PHENACETIN TO REACTIVE INTERMEDIATES

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(*Received* 19 *March* 1977; *accepted* 4 *October* 1977)

Abstract—The *N*-*O*-glucuronide of [^{14}C]acetyl-*N*-hydroxyphenacetin is sufficiently stable to purify, but slowly breaks down in aqueous solutions to a reactive intermediate that can covalently bind to protein. When the pure compound was incubated in Tris buffer, pH 7.4, at 37°, it decomposed with a half-life of about 8.7 hr to the following compounds: phenacetin, 2-hydroxyphenacetin glucuronide, acetamide and acetaminophen. On addition of glutathione to the systems and allowing the reactions to go to completion, a glutathione–acetaminophen conjugate was formed at the expense of acetamide and acetaminophen; the fraction converted to phenacetin or to the 2-hydroxyphenacetin glucuronide was unchanged. On addition of ascorbic acid to the system and allowing the reactions to go to completion, the fraction converted to acetaminophen was increased at the expense of acetamide; the fractions converted to phenacetin and 2-hydroxyphenacetin glucuronide, however, were again unchanged. When the glucuronide was incubated with bovine serum albumin, covalent binding to the protein occurred at the expense of acetaminophen and acetamide; again, the fraction of the glucuronide converted to phenacetin and 2-hydroxyphenacetin glucuronide was unchanged. Moreover, the covalent binding could be partially prevented by addition of ascorbic acid or glutathione. Since there is formation of covalently bound material, the glutathione conjugate and acetaminophen appear to be interrelated; it seems likely that they are formed from a common intermediate, possibly acetylimidoquinone. However, the data suggest that the formation of phenacetin and 2-hydroxyphenacetin glucuronide occurs by different mechanisms. The *N*-*O*-sulfate of [^{14}C]acetyl-*N*-hydroxyphenacetin also breaks down to a reactive intermediate that has properties similar to those of the reactive intermediate formed from the *N*-*O*-glucuronide and thus may also be *N*-acetylimidoquinone. By contrast, the relative ability of various nucleophiles to prevent the covalent binding of the reactive intermediate formed from the *N*-*O*-sulfate of 2-acetylaminofluorene to protein differs from the relative ability of the nucleophiles to prevent the covalent binding of the reactive intermediate of either the *N*-*O*-sulfate or the *N*-*O*-glucuronide of phenacetin, suggesting that the relative rates at which these intermediates combine with the different macromolecules may differ markedly.

N-hydroxylation has been implicated in the toxicities observed with several of the *N*-acetylarylamines [1–3]. For example, the centrilobular hepatic necrosis caused by large doses of acetaminophen in man or experimental animals has been attributed to an arylating metabolite arising through *N*-hydroxylation [1, 4–8]. Moreover, the carcinogenicity of several of the polycyclic *N*-acetylarylamines such as 2-acetylaminofluorene (2-AAF) is believed to be mediated through *N*-hydroxy metabolites [2, 3]. *N*-hydroxy-2-acetylaminofluorene is believed to be further metabolized by a sulfotransferase to its *N*-*O*-sulfate ester. This sulfate ester is capable of reacting with certain groups of DNA, a reaction which may partially account for the carcinogenic effect of *N*-hydroxy-2-acetylaminofluorene [2, 9].

Although phenacetin, which is converted to acetaminophen in animals, has not been shown to be carcinogenic in animals, it does produce a centrilobular hepatic necrosis in animals [1]. In addition, human abusers of this drug show a high incidence of nephropathy and renal pelvic tumors [10, 11].

Although the mechanisms of these toxic effects of phenacetin are unknown, it seems plausible that they may be mediated by arylating metabolites. There is evidence that arylating metabolites of phenacetin may be formed by at least three different mechanisms. (1) Phenacetin may be converted to acetaminophen which in turn is activated to an arylating metabolite [1]. (2) Phenacetin may be oxidized directly to an arylating metabolite by a hepatic cytochrome P-450, possibly through the formation of phenacetin-3,4-epoxide [12]. (3) Phenacetin may be *N*-hydroxylated to form the stable metabolite, *N*-hydroxyphenacetin, which in turn is converted to its unstable *N*-*O*-sulfate and *N*-*O*-glucuronate derivatives [13, 14].

Recent studies have revealed that the *N*-*O*-glucuronate of *N*-hydroxyphenacetin is far more stable than the *N*-*O*-sulfate derivative and indeed can be isolated and purified [14]. In the present study, we have obtained further proof for the formation of the *N*-*O*-glucuronide of phenacetin, have assessed various substances as trapping agents of the reactive intermediate, and have characterized some of its breakdown products.

MATERIALS AND METHODS

Chemicals. The *N*-hydroxy-*N*-arylacetamides were synthesized by reduction of the nitroanalogues in the presence of zinc to the hydroxylamine, followed by acetylation with acetyl chloride as previously described [8, 13, 15, 16]. For the preparation of ^{14}C-labeled *N*-hydroxy-*N*-arylacetamides, [^{14}C]acetyl chloride (New England Nuclear, Boston, MA, U.S.A.) was used. For the synthesis of [^{14}C]ethoxy-*N*-hydroxyphenacetin, *p*-nitrophenol was ethylated with [1-^{14}C]ethyl iodide (New England Nuclear, Boston, MA, U.S.A.)[17], and the resulting [^{14}C]ethoxy-*p*-nitrophenetole was used for the synthesis of *N*-hydroxyphenacetin. Glutathione, ascorbic acid, phosphoadenosine phosphate (PAP), UDP-glucuronate, Triton X-100 and *p*-nitrophenylsulfate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine serum albumin (BSA) was obtained from Armour Pharmaceuticals, Chicago, IL, U.S.A. [D-Glucuronic acid-^{14}C-U]-UDP glucuronic acid was a product of New England Nuclear. Thin-layer chromatographic plates were obtained from Analtech, Newark, DE, U.S.A. All other chemicals were of the purest grade available.

Rats and enzyme preparations. Adult male Sprague–Dawley rats (Charles River CDS, Wilmington, MA), 200–300 g, had free access to food and water. The animals were decapitated and the livers removed and homogenized in a Potter–Elvehjem homogenizer with 3 vol. of 0.15 M KCl, buffered with 20 mM Tris–HCl, pH 7.4. Microsomes and the postmicrosomal supernatant fraction were obtained as described previously [14]. Microsomes were treated with Triton X-100 to activate microsomal UDP-glucuronyl transferase as previously described [18]. Prior to use, the postmicrosomal supernatant fraction was chromatographed over Sephadex G-25 (45 × 2.5 cm volume) with 50 mM sodium phosphate buffer, pH 7.4, as the eluant to remove glutathione and other low molecular weight substances [14].

Protein determination. Protein was determined according to the method of Lowry *et al.* [19] using bovine serum albumin as a standard.

Sulfation of N*-hydroxyarylacetamides.* The incubation medium (1 ml) contained (final concentrations) 5 mM $MgCl_2$; 20 mM sodium phosphate buffer, pH 7.4; 5 mM Tris–HCl buffer, pH 7.4; 10 mM *p*-nitrophenylsulfate; 20 μM PAP; 0.5 mM [*N*-acetyl-^{14}C]*N*-hydroxyarylacetamide substrate; and about 4 mg/ml of postmicrosomal supernatant protein. The mixtures were incubated for 5–10 min at 37°C under air. The reaction was terminated by addition of an equal volume of methanol, and the covalent binding of the labeled substrates was determined as previously described [5, 14].

Synthesis of the N-O*-glucuronide conjugate of* N*-hydroxyphenacetin.* The conjugate was synthesized enzymatically in a 25-ml incubation mixture containing: 5 mM $MgCl_2$, 2 mM UDPGA; 45 mM Tris–HCl buffer, pH 7.4; 0.5 mM *N*-hydroxyphenacetin; and about 4 mg/ml of microsomal protein (treated with Triton X-100). After incubation of the mixture for 60 min at 37°, protein was precipitated with 2 vol methanol and removed by centrifugation. The methanol was evaporated with a stream of nitrogen gas and the aqueous layer was lyophilized. The residue was dissolved in a minimal volume of water applied to four 20 × 20 cm thin-layer chromatographic plates (Avicel F, 250 μm). The chromatography was developed using the solvent, *l*-propanol–0.4 M ammonium hydroxide (80:20). The phenacetin *N*-*O*-glucuronide (R_f 0.50) was visualized by u.v. light, scraped into a tube and eluted overnight with methanol–water (80:20) at 4°. The methanol–water phase was evaporated under a stream of nitrogen gas and the residue stored at −40°. Owing to its instability, the compound could not be stored for more than 2 days. The stability of the compound could be prolonged by addition of $MgCl_2$. Immediately before use, the compound was dissolved in water. When the breakdown products of phenacetin *N*-*O*-glucuronide were quantitated, the u.v. spot was immediately extracted from the thin-layer plate with methanol–0.16 mM Tris–HCl (80:20), pH 7.4, at 4°, followed by removal of the methanol by flash evaporation. The purity of the phenacetin *N*-*O*-glucuronide was greater than 98 per cent as determined by thin-layer chromatography (t.l.c.) on Avicel F, using *l*-propanol–0.4 mM ammonium hydroxide (80:20) as the developing solvent.

Thin-layer chromatographic isolation of the breakdown products of the N*-hydroxy conjugate of* N*-hydroxyphenacetin.* The glutathione conjugate from incubations containing the *N*-*O*-glucuronide of [^{14}C]*N*-hydroxyphenacetin was isolated on a 5 × 20 cm Avicel F (250 μm) thin-layer chromatographic plate using *n*-propanol–0.4 M ammonium hydroxide (80:20) as the developing solvent. In this system, the material covalently bound to protein did not move from the origin, the glutathione conjugate had an R_f of 0.05, phenacetin *N*-*O*-glucuronide had an R_f of 0.5, 2-hydroxyphenacetin glucuronide had an R_f of 0.4, acetamide had an R_f of 0.7 and the aglycones had an R_f of 0.85. The radioactive substances were scraped into scintillation counting vials and eluted by the addition of 0.2 ml water. Fifteen ml of a scintillation fluiding containing 4 g [2.5-bis-2-(5-tertbutylbenzoxazolyl)-thiophene] (BBOT), 2.0 l toluene, 280 g naphthalene, and 1.4 l methyl cellosolve was added and the substances were quantitated using a Searle Mark III liquid scintillation spectrometer. Covalent binding was also determined by exhaustive solvent extraction of the precipitated protein as described by Jollow *et al.* [5].

The aglycones were isolated using a two-dimensional thin-layer chromatographic system originally developed for the isolation of *N*-hydroxyphenacetin [13]. A sample of the incubate was applied to one corner of a 20 × 20 cm Silica gel GF (250 μm) thin-layer chromatographic plate along with 100 μg of each of the following unlabeled reference compounds: phenacetin, acetaminophen, *N*-hydroxyphenacetin and 2-hydroxyphenacetin. The thin-layer chromatographic plate was then developed in the first dimension with ether–hexane (85:15) (solvent system 1) as the developing solvent. In the first dimension the R_f values of the following compounds were: *N*-hydroxyphenacetin (R_f 0.29), phenacetin (R_f 0.25), acetaminophen (R_f

0.16), 2-hydroxyphenacetin (R_f 0.34), 4-aminophenol (R_f 0.39) and phenetidine (R_f 0.68). For the second dimension the chromatograph was developed with ethyl acetate–NH_4OH (99:1) (solvent system 2); the compounds had the following R_f values: *N*-hydroxyphenacetin (R_f 0.23), phenacetin (R_f 0.67), acetaminophen (R_f 0.40), 2-hydroxyphenacetin (R_f 0.53), 4-aminophenol (R_f 0.63) and phenetidine (R_f 0.81). The radiolabeled substances were detected by u.v. light, scraped into scintillation vials and quantitated by liquid scintillation spectrometry.

Incubation conditions for studying the breakdown of the N-O-*glucuronide of* N-*hydroxyphenacetin.* The composition of the incubation medium used to study the rearrangement products of the *N-O*-glucuronide of *N*-hydroxyphenacetin was: 5 mM $MgCl_2$; 40 mM Tris–HCl buffer, pH 7.4; and approximately 0.5 mM of the *N-O*-glucuronide of [^{14}C]*N*-hydroxyphenacetin. A drop of toluene was added to some samples to prevent bacterial growth. The standard incubation time was 24 hr at 37°. A sample of the incubation mixture was applied to the thin-layer plates at the end of the incubation and was analyzed as described above.

RESULTS

*Formation of phenacetin-*N-O-*glucuronide.* Triton-treated rat liver microsomes were incubated with: (1) unlabeled UDP-glucuronate and [^{14}C]acetyl-labeled *N*-hydroxyphenacetin, (2) unlabeled UDP-glucuronate and [^{14}C]ethoxy-labeled *N*-hydroxyphenacetin, or (3) [^{14}C]UDP-glucuronide and unlabeled *N*-hydroxyphenacetin. Isolation of the glucuronides formed in these mixtures revealed that they contained an *N*-acetyl, an *O*-ethyl and a glucuronate group in equivalent amounts. Thus, the glucuronide was neither acetaminophen glucuronide nor phenetidine-*N-O*-glucuronide and presumably was the *N-O*-glucuronide of *N*-hydroxyphenacetin.

Covalent binding of the reactive intermediate of phenacetin N-O-*glucuronide.* When [^{14}C]acetyl-labeled phenacetin *N-O*-glucuronide (500 nmoles) was incubated with bovine serum albumin (10 mg/ml), pH 7.4, for 24 hr at 37°, the compound (85 nmoles) covalently bonded to albumin as judged from the radioactivity that was associated with protein. When the ^{14}C-label was in either the *O*-ethyl or the *N-O*-glucuronate group, however, no radioactivity was covalently bound to albumin. These data indicate that during the conversion of phenacetin *N-O*-glucuronide to the reactive intermediate, the glucuronate and the *O*-ethyl groups were lost, whereas the *N*-acetyl group was retained. Similarly, during the generation of the *N-O*-sulfate conjugate of *N*-hydroxyphenacetin, radioactivity was covalently bound to protein when ^{14}C was in the *N*-acetyl group but not when the ^{14}C was in the *O*-ethyl group.

Analysis of the incubation mixture by thin-layer chromatography after incubation for 24 hr at 37° revealed the presence of phenacetin, acetaminophen, acetamide and the 2-hydroxyglucuronide of 2-hydroxyphenacetin. No unconjugated 2-hydroxyphenacetin, or *N*-hydroxyphenacetin was found. Similarly, no phenetidine or *p*-aminophenol could be found after incubation; after exposure of the plates to the air, as little as 5 nmoles of these substances was detected as black polymerized products. Thus, the *N*-acetyl group is not transferred directly to the protein by transacetylation to any significant extent.

Half-life of breakdown of phenacetin in N-O-*glucuronide.* The half-life of phenacetin *N-O*-glucuronide was determined from the half-time of formation of the ethyl acetate-extractable materials, phenacetin and acetaminophen. In the standard incubation the half-life was approximately 8.7 hr and was not dependent upon the concentration of the phenacetin *N-O*-glucuronide; thus the breakdown of the glucuronide was spontaneous and not a biomolecular reaction. We calculate that the reaction would be about 85 per cent complete at the end of 24 hr and about 97 per cent complete at the end of 45 hr.

Effect of nucleosides and various amino acids on covalent binding of N-O-*sulfate of* N-*hydroxyphenacetin,* N-O-*glucuronide of* N-*hydroxyphenacetin and* N-O-*sulfate of* N-*hydroxy-2-acetylaminofluorene.* In order to determine the kinds of groups in protein and other macromolecules that combine with the reactive intermediate, [^{14}C]acetyl-labeled *N*-hydroxyphenacetin and *N*-hydroxy-2-acetylaminofluorene were incubated with liver-soluble fraction and a 3-phosphoadenosine-5′-phosphosulfate (PAPS)-generating system in the presence of various substances, as described in Materials and Methods. As shown in Table 1, the covalent binding of the reactive intermediate from the *N-O*-sulfate conjugate of 2-acetylaminofluorene was inhibited by both adenosine (2 mM) and guanosine (2 mM), in agreement with previously reported data [9]. These nucleosides, however, had little effect on the covalent binding of the reactive intermediate formed from the conjugates of *N*-hydroxyphenacetin. In contrast, glutathione (0.25 mM) almost completely inhibited the covalent binding of the reactive intermediate of the conjugates of *N*-hydroxyphenacetin but decreased the covalent binding of the sulfate of *N*-hydroxy-2-acetylaminofluorene by only 13 per cent. Cysteine (0.25 mM) inhibited the covalent binding of the reactive intermediate produced by sulfation of *N*-hydroxyphenacetin to a greater extent than it inhibited the covalent binding of the reactive intermediate produced from the phenacetin *N-O*-glucuronide or the reactive intermediate produced by sulfation of *N*-hydroxy-2-acetylaminofluorene. With methionine (20 mM), however, inhibition of covalent binding was observed with the sulfate of *N*-hydroxy-2-acetylaminofluorene but not with that of *N*-hydroxyphenacetin. Neither the covalent binding of the reactive intermediate derived from the sulfate conjugate of *N*-hydroxy-2-acetylaminofluorene nor that of the conjugates of *N*-hydroxyphenacetin was inhibited by any of the other amino acids tested: tryptophan, alanine, glutamine, glutamic acid, lysine, histidine, tyrosine and proline. However, ascorbic acid inhibited the covalent binding of all three compounds.

Glutathione conjugate derived from phenacetin

Table 1. Effect of sulfur-containing amino acids, nucleosides and ascorbic acid on the covalent binding of the *N*-*O*-sulfate and the *N*-*O*-glucuronide of *N*-hydroxyphenacetin, and the *N*-*O*-sulfate of *N*-hydroxy-2-acetylaminofluorene*

Compound	Concn (mM)	Per cent inhibition of covalent binding to protein		
		N-hydroxyphenacetin		*N*-hydroxy-2-acetyl-aminofluorene
		N-*O*-sulfate	*N*-*O*-glucuronide	*N*-*O*-sulfate
Glutathione	0.25	98	98	13
Cysteine	0.25	95	47	30
Methionine	20	4	20	87
Adenosine	2.0	0	0	15
Uridine	2.0	0	0	0
Guanosine	2.0	0	0	60
Cytidine	2.0	0	0	0
Ascorbate	0.5	75	67†	70

* When the effect on covalent binding of the *N*-*O*-sulfate of both *N*-hydroxyphenacetin and *N*-hydroxy-2-AAF was investigated, the *N*-*O*-sulfate was generated *in situ* with a postmicrosomal supernatant fraction (5 min at 37°) and the covalent binding was determined in the same incubation in the presence or absence of the inhibitor. In the presence of no inhibitor, 4.2 nmoles/mg of postmicrosomal supernatant was bound with *N*-hydroxyphenacetin and 3.9 nmoles/mg of postmicrosomal supernatant was bound with *N*-hydroxy-2-acetylaminofluorene. With the *N*-*O*-glucuronide of *N*-hydroxyphenacetin a stored preparation was used (1 ml containing 0.5 μmole) and was incubated with bovine serum albumin (13 mg/ml) for 2 hr at 37°. In the absence of inhibitor, 1.1 nmoles/mg of BSA was bound with phenacetin *N*-*O*-glucuronide.

† Ascorbate concentration, 1.0 mM.

Table 2. Effect of varying incubation conditions on the breakdown of phenacetin *N*-*O*-glucuronide*

Addition to incubation	Acet-aminophen (%)	Phenacetin (%)	Acetamide (%)	2-Hydroxy-phenacetin glucuronide (%)	Glutathione conjugate (%)	Covalent binding (%)
Expt. 1						
None	35.9	19.3	17.7	27.1		
	35.3	19.7	17.5	27.4		
Bovine serum albumin	21.2	20.2	12.5	27.3		18.8
	20.3	20.2	11.9	27.8		19.9
Ascorbic acid	47.1	20.2	6.8	25.9		
	46.6	20.1	7.1	26.2		
Glutathione	30.5	20.1	9.5	27.8	11.9	
	30.4	19.7	10.7	28.3		
Expt. 2						
Bovine serum albumin	18.4					13.8
Bovine serum albumin and glutathione	18.9				18.0	1.7
Bovine serum albumin and ascorbic acid	38.6					4.6

* In Expt. 1, the phenacetin *N*-*O*-glucuronide (374 nmoles) (500 dis/min/nmole) (> 98 per cent pure as determined by thin-layer chromatography immediately before use) was incubated for 45 hr at 37° in 1 ml of 40 mM Tris buffer plus 5 mM $MgCl_2$. The concentrations of the addition to the incubation mixtures were bovine serum albumin (10 mg), ascorbic acid (1 μmole) and glutathione (1 μmole). At the end of this incubation, phenacetin and acetaminophen were assayed on a 250-μm, 20 × 20 cm Silica gel GF as described in Materials and Methods (a 100-μl fraction was used). The water-soluble products were assayed by spotting 100 μl of the incubate on an Avicel F plate (solvent *n*-propanol–0.4 M ammonium hydroxide) as described in Materials and Methods. These plates were then scraped in 0.5-cm segments and the compounds quantitated by liquid scintillation spectrometry. There was an average of 92.9 per cent of the radioactivity recovered; the percentages are based upon recovered radioactivity. Each incubation mixture was run in duplicate and the data from each incubation mixture are shown. In Expt. 2, the phenacetin *N*-*O*-glucuronide (500 nmoles) from a stored preparation was incubated 24 hr under conditions similar to those in Expt. 1. The glutathione conjugate was quantitated by t.l.c. after precipitation of the protein with methanol. In this experiment acetaminophen was quantitated as described in Materials and Methods. The other breakdown products were not quantitated.

N-O-*glucuronide*. The finding that glutathione almost completely prevented the covalent binding of the reactive intermediate of phenacetin *N-O*-glucuronide raised the possibility that the reaction of glutathione with the intermediate to form a glutathione conjugate resulted in a decrease in the steady state concentration of the intermediate and thereby a decrease in the amount that reacts with protein. In accord with this view, the amount of glutathione conjugate formed was similar to the amount that was covalently bound to serum albumin (Table 2). Moreover, when glutathione and bovine serum albumin were incubated with phenacetin *N-O*-glucuronide, covalent binding was decreased (Tables 1 and 2) with a concomitant formation of a glutathione conjugate (Table 2).

Since it seemed likely that the covalently bound species and the glutathione conjugate were derived from the same chemically reactive intermediate, studies were performed to identify the conjugate. After incubation of phenacetin *N-O*-glucuronide with glutathione for 24 hr at 37° in buffer, pH 7.4, the glutathione conjugate was isolated and treated with Raney nickel, which reductively cleaves glutathione from the conjugate to yield a product that can be analyzed by mass spectrometry [12].

As shown in Fig. 1, the mass spectrum indicated that the product was *p*-hydroxyacetanilide (acetaminophen). Thus, the reactive intermediate had retained the acetyl group but had lost both the ethyl group and glucuronide moiety of phenacetin *N-O*-glucuronide, a fact that is consistent with the finding that the isotopically-labeled acetyl group was covalently bound to protein but that the labeled ethyl group was not. These results, therefore, support the view that the chemically reactive intermediate is a de-ethylated substance that reacts with either glutathione or protein. In a control experiment, acetaminophen did not spontaneously covalently bind to protein when incubated for 45 hr.

Acetaminophen as a breakdown product of phenacetin N-O-*glucuronide*. When phenacetin *N-O*-glucuronide was incubated for 45 hr at 37° in 40 mM Tris plus 5 mM $MgCl_2$ buffer, pH 7.4, about 35.6 per cent was converted to acetaminophen (Table 2). The addition of serum albumin (10 mg/ml) or glutathione (1 mM), however, decreased the amount of acetaminophen formed, but the addition of ascorbic acid (1 mM) increased the formation of acetaminophen. These findings suggested that covalent binding, glutathione conjugation, and acetaminophen formation were formed, at least in part, from a common intermediate. The finding that ascorbic acid increases the formation of acetaminophen and decreases the amount of covalent binding suggested that the reactive intermediate is an easily reduced substance, possibly *N*-acetylimidoquinone.

Whether acetaminophen may be formed by an additional mechanism is unresolved. Increasing the concentration of glutathione (from 1 to 100 mM) increased the formation of glutathione conjugate and decreased slightly the formation of the acetaminophen (Fig. 2). Thus, it appears that a part of

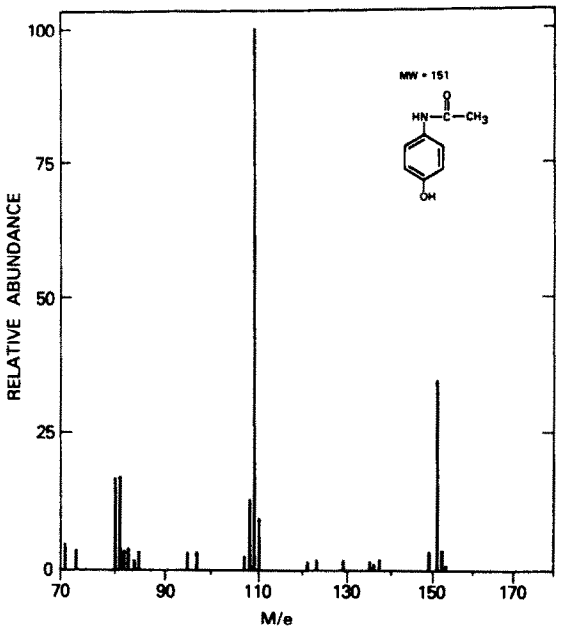

Fig. 1. Mass spectrum of acetaminophen after Raney nickel treatment of glutathione conjugate. Phenacetin *N-O*-glucuronide was isolated from a 100-ml incubation as described in Materials and Methods and incubated for 24 hr at 37° with 1 mM glutathione. The glutathione conjugate was isolated as previously described [12] and treated with Raney nickel to reductively cleave the glutathione from the ring. The acetaminophen was subsequently isolated by t.l.c. on one 5 × 20 cm, 250-μm Silica gel GF plate in solvent system 1 and subjected to mass spectrometry as previously described [13].

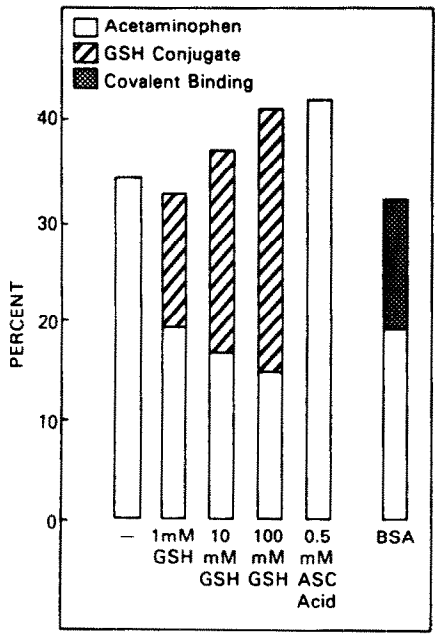

Fig. 2. Effect of glutathione on acetaminophen and glutathione conjugate formation from phenacetin *N-O*-glucuronide. The *N-O*-glucuronide of *N*-hydroxyphenacetin was incubated at 37° for 24 hr in Tris–HCl buffer, pH 7.4. Glutathione (GSH) was added in the final concentrations as indicated. Also ascorbic acid (ASC acid) (0.5 mM) and bovine serum albumin (BSA) were added in separate incubations. The data are reported as the per cent of the *N-O*-glucuronide converted to acetaminophen, the GSH conjugate, and bound covalently to BSA.

the acetaminophen may be formed either by reduction of the chemically reactive intermediate by glutathione or by a mechanism that does not involve the chemically reactive intermediate.

Acetamide from phenacetin N-O-*glucuronide.* From the incubation of [^{14}C]acetyl-phenacetin *N-O*-glucuronide, a water-soluble compound, was found with the R_f characteristic of authentic [^{14}C]acetamide. To ascertain if this compound was acetamide, phenacetin *N-O*-glucuronide was incubated with buffer overnight and the ^{14}C-material isolated by thin-layer chromatography as described in Materials and Methods. This ^{14}C-labeled material was then mixed with authentic unlabeled acetamide and recrystallized four times. As shown in Table 3, the specific activity of the radiolabeled compound remained constant during the recrystallizations, confirming the view that the compound was acetamide.

The acetamide probably arises by hydrolysis of the proposed reactive intermediate, acetylimidoquinone, since, in the presence of bovine serum albumin or glutathione where the respective adducts are formed from the reactive intermediate, acetamide is decreased (Table 2). Moreover, ascorbic acid, which reduces the acetylimidoquinone to acetaminophen, drastically decreases the amount of acetamide. The nature of the hydrolysis product containing the ring was not determined since this portion of the molecule did not contain a radiolabel.

Formation of phenacetin from phenacetin N-O-*glucuronide.* During the incubation of phenacetin *N-O*-glucuronide in Tris buffer, about 20 per cent of the conjugate is converted to phenacetin. However, the addition of serum albumin, glutathione or ascorbic acid did not significantly change the amount of phenacetin formed, indicating that phenacetin was not formed by way of the chemically reactive intermediate, but was probably derived by another mechanism, possibly an intramolecular rearrangement of the phenacetin *N-O*-glucuronide, resulting in phenacetin and an oxidized product of glucuronic acid. Moreover, when a sample of the phenacetin *N-O*-glucuronide was incubated with 75 per cent methanol, under conditions identical to those described in Table 2, there was a 15 per cent conversion to phenacetin, and only trace amounts of acetaminophen were detected, further indicating the two pathways were different.

Table 3. Constant specific activity of [^{14}C]acetamide product during sequential crystallization*

Crystallization and solvent	Acetamide (g)	Specific activity (dis./min/g)
Initial solution	2.00	11,288
First crystallization (*n*-propranol–heptane, 50:50)	1.43	11,762
Second crystallization (*n*-propranol–heptane, 50:50)	0.97	12,126
Third crystallization (ethyl acetate)	0.78	10,645
Fourth crystallization (ethyl acetate)	0.55	10,556

* Approximately 500 nmoles (500 dis/min/nmole) [^{14}C]-phenacetin-*N-O*-glucuronide was incubated with Tris buffer for 24 hr. [^{14}C]acetamide was subsequently isolated on four 250-μm, 20 × 20 cm Avicel F plates [solvent: *n*-propanol–0.4 M ammonium hydroxide (80:20)]. A band with $R_f = 0.7$ was removed by extraction with methanol. The material was flash evaporated, mixed with 2 g of authentic unlabeled acetamide and crystallized in the appropriate solvent. After crystallization the samples were weighed and the radioactivity was determined by liquid scintillation spectrometry. Each specific activity is the average of duplicate determinations.

2-Hydroxyphenacetin glucuronide as a rearrangement product of N-*hydroxyphenacetin glucuronide.* 2-Hydroxyphenacetin glucuronide was a rearrangement product of phenacetin *N-O*-glucuronide comprising approximately 27 per cent of the breakdown products under all incubation conditions. The product, isolated after incubation of *N-O*-glucuronide with buffer, was cleaved with β-glucuronidase. The aglycone after hydrolysis had the same R_f as 2-hydroxyphenacetin in solvent systems 1 and 2 (see Materials and Methods) and gave a mass spectrum identical to that of 2-hydroxyphenacetin (Fig. 3). Since the amount of this product was approximately the same in all incubations, its formation appears to be unrelated to the formation of the reactive intermediate, acetylimidoquinone. Moreover, when a sample of the phenacetin *N-O*-glucuronide was incubated with 75% methanol about 15% of it was converted to phenacetin, but no 2-hydroxyphenacetin glucuronide was detected, indicating the isomerization pathway is different from that leading to phenacetin. The mechanism of this isomerization would presumably be similar to the rearrangement of *N*-acetoxyphenacetin to 2-acetoxyphenacetin in nonaqueous solvents reported by Calder *et al.* [15].

Products formed from phenacetin N-O-*sulfate.* When [^{14}C]acetyl-labeled *N*-hydroxyphenacetin was incubated with liver-soluble fraction and a PAPS-generating system, considerable amounts of the radioisotope were covalently bound to protein or converted to acetaminophen (Fig. 4). However, very little of the substrate was converted to phenacetin. Moreover, addition of ascorbic acid markedly decreased the covalent binding of the radioisotope and increased the formation of acetaminophen. This concentration of ascorbic acid (1 mM) had no inhibitory effect on sulfation of *N*-hydroxyphenacetin as measured with the *p*-nitrophenylsulfate-coupled assay [14]. Thus, the chemically reactive intermediate formed from the *N-O*-sulfate is similar if not identical to that formed from the *N-O*-glucuronide of *N*-hydroxyphenacetin.

DISCUSSION

Nery [20] has reported that a small fraction of a dose of [^{14}C]ethyl-labeled phenacetin is converted to *N*-acetyl-*S*-[^{14}C]ethyl cysteine and that a small portion of [^{3}H]acetyl-labeled phenacetin is metabolized to [^{3}H]acetamide in rats. Moreover, he reported that the amount of [^{3}H]acetamide excreted into urine was about the same as the amount of

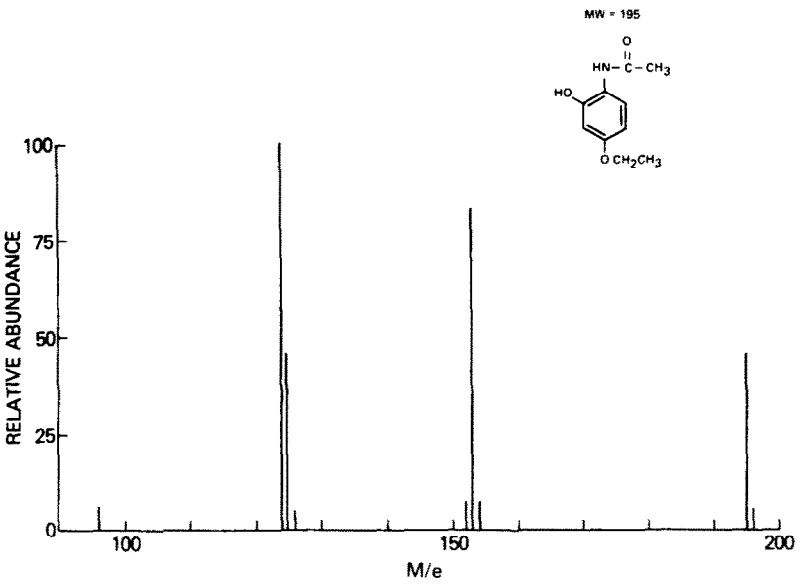

Fig. 3. Mass spectrum of 2-hydroxyphenacetin after β-glucuronidase treatment of glucuronide breakdown product. Approximately 500 nmoles (500 dis/min/nmole) [^{14}C]phenacetin *N*-*O*-glucuronide was incubated with Tris buffer for 24 hr. The breakdown products were isolated on four 250-μm, 20 × 20 cm Avicel F plates [solvent: *n*-propranol–0.4 M ammonium hydroxide (80:20)]. A band with R_f 0.4 was removed by extraction with 0.1 M sodium acetate, pH 5.0 (21 ml). This extract was divided into two fractions of 10 ml each, and to one was added 40 mg β-glucuronidase (*Helix pomatia*) (10,000 units). Each was incubated 15 hr, after which time each was extracted with ethyl acetate (20 ml). No radioactivity was extracted from the compound with no enzyme, and 75 per cent of the radioactivity was extracted from the incubation mixture containing β-glucuronidase. This radiolabeled compound had the same R_f of 2-hydroxyphenacetin in solvents 1 and 2. The compound was isolated in solvent 1 and subjected to electron impact mass spectrometry under conditions similar to those described for acetaminophen. The mass spectrum shown is identical to that of authentic 2-hydroxyphenacetin (Aldrich, Milwaukee, Wisconsin).

hydroquinone excreted as its sulfate and glucuronide conjugates. He also reported that a small portion of the dose of phenacetin was excreted as a sulfoxide of a mercapturic acid that contained both *N*-acetyl and *O*-ethyl groups. On the basis of these findings, he postulated that *N*-hydroxyphenacetin could be converted to *N*-*O*-sulfate and *N*-*O*-glucuronide conjugates and that the formation of these conjugates could lead to the formation of most of the urinary metabolites of phenacetin. According to his view, the conjugates would spontaneously decompose to form an intermediate having a carbonium ion in the ortho position. This intermediate could then react with glutathione to form phenacetin-*S*-glutathione, which would be excreted ultimately as the mercapturic acid sulfoxide. Alternatively, the intermediate could lose its ethyl group and form *N*-acetylimidoquinone, which in turn could be either reduced to acetaminophen or hydrolyzed to form acetamide and *p*-quinone. As pointed out by Calder *et al.* [21], however, *N*-acetylimidoquinone is a chemically reactive substance that not only undergoes hydrolysis but also reacts with various nucleophilic substances such as *S*-methylcysteine and *N*-acetylmethionine to form 3-methylthioacetaminophen.

The evidence presented in this paper supports the view that the major chemically reactive intermediate formed from sulfation or glucuronidation of *N*-hydroxyphenacetin is acetylimidoquinone (Fig. 5). The finding that ^{14}C was covalently bound when phenacetin *N*-*O*-glucuronide was labeled in the *N*-acetyl group but not when it was labeled in the ethoxy group led to the conclusion that the covalently bound substance could be either acetate or a de-ethylated breakdown product of *N*-hydroxyphenacetin. However, other evidence indicated that the reactive intermediate was the de-ethylated derivative, *N*-acetylimidoquinone. First, attempts to discover deacetylated breakdown products of phenacetin *N*-*O*-glucuronide failed to reveal the formation of either phenetidine or *p*-aminophenol. Second, both the glutathione conjugate and at least a part of the acetaminophen appeared to be formed from the same chemically reactive intermediate that becomes covalently bound to protein (Table 2). Third, the glutathione conjugate was a derivative of acetaminophen rather than phenacetin. Fourth, ascorbic acid, which decreased covalent binding of [^{14}C]acetyl-labeled phenacetin *N*-*O*-glucuronide, increased the formation of acetaminophen, suggesting that the reactive intermediate was an easily reduced de-ethylated product (Table 2). Fifth, acetamide formation, which would be formed from acetylimidoquinone, was highest in incubations where acetylimidoquinone could not covalently bind to protein, conjugate with glutathione or be readily reduced to acetaminophen. On the other hand, the finding that ascorbic acid does not significantly affect the formation of phenacetin in the system indicates that phenacetin was not derived from the chemically reactive intermediate but was

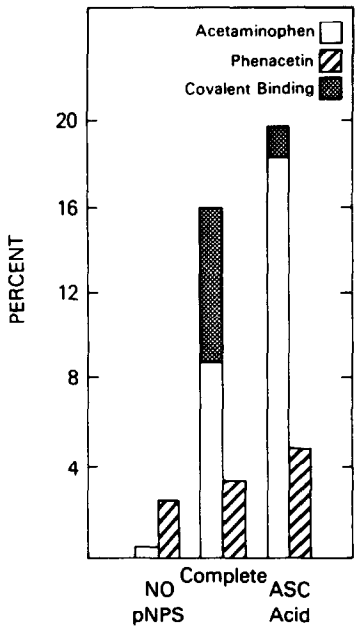

Fig. 4. Effect of ascorbic acid on acetaminophen formation and covalent binding of the *N*-*O*-sulfate of *N*-hydroxyphenacetin. *N*-hydroxyphenacetin was incubated with the postmicrosomal supernatant fraction from rat liver in the absence or presence of *p*-nitrophenylsulfate (10 mM) under conditions described in Materials and Methods for the sulfation assay. PAP was always present and ascorbic acid (0.5 mM) was present when indicated. Incubation was for 60 min at 37° after which time methanol was added to stop the reaction, and the supernatant was analyzed for phenacetin, acetaminophen and covalently bound material. The amounts are expressed as the percentage of *N*-hydroxyphenacetin added to the incubation.

possibly formed from an intramolecular rearrangement of the phenacetin *N*-*O*-glucuronide that is not mediated by the chemically reactive intermediate. Also, the fact that bovine serum albumin, ascorbic acid or glutathione did not affect the formation of the 2-hydroxyphenacetin glucuronide indicates this isomerization is independent of the formation of acetylimidoquinone.

Because the covalently bound species and the glutathione conjugate do not contain an ethoxy group, and because ascorbic acid did not increase the formation of phenacetin, it appears that the *p*-ethoxy-*N*-acetylimidoquinone postulated by Nery [20], if formed at all, undergoes *O*-de-ethylation at pH 7.4 very rapidly. Indeed, even when the reactive intermediate was generated by way of the *N*-*O*-sulfate conjugate, very little phenacetin was formed in the presence of ascorbic acid (Fig. 4).

Acetylimidoquinone also undergoes hydrolysis to form acetamide and presumably quinone as postulated by Nery [20]. Although the formation of quinone and its covalent binding to protein would be undetected in this study because it lacks the labeled *O*-ethyl and *N*-acetyl groups, increasing the concentration of glutathione presumably would decrease the formation of quinone and acetamide as well as the formation of acetaminophen by diverting the acetylimidoquinone toward the glutathione conjugate.

The finding that acetaminophen was a breakdown product of *N*-*O*-glucuronide and *N*-*O*-sulfate conjugates supports the postulate of Nery that these conjugates could lead to a readily reducible reactive intermediate. However, the finding that glutathione does not completely block the formation of acetaminophen suggests either that glutathione can reduce the reactive intermediate as well as form a conjugate or that acetaminophen may also be formed by a mechanism that is not mediated by the

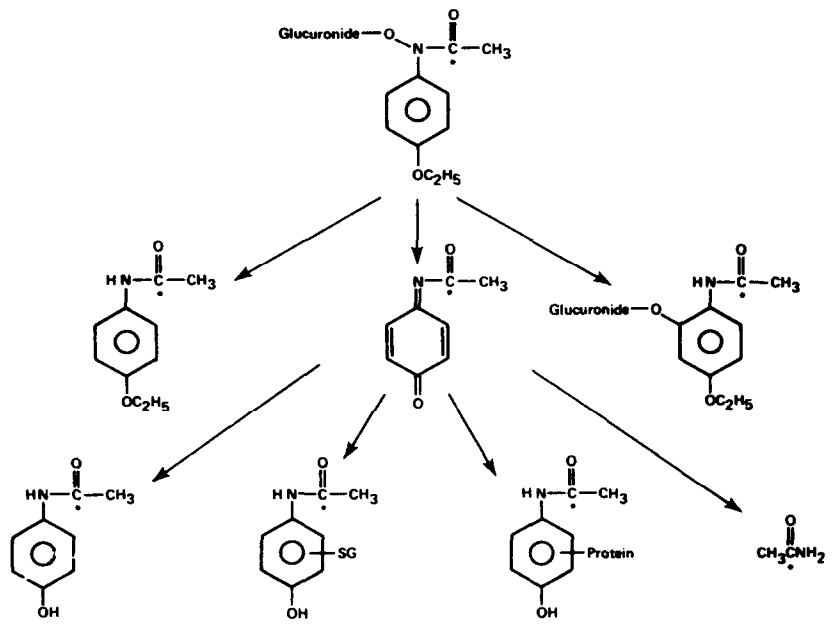

Fig. 5. Proposed mechanism for breakdown of phenacetin *N*-*O*-glucuronide.

reactive intermediate. Moreover, the implication that acetaminophen is formed from phenacetin solely by way of an *N*-hydroxylation reaction seems unsubstantiated because Hinson *et al.* [12] have shown that the rate of *O*-de-ethylation of phenacetin by hamster liver microsomes is an order of magnitude faster than its rate of *N*-hydroxylation [13].

Although Calder *et al.* [21] showed that *N*-acetoxyphenacetin can react with methionine at boiling water temperatures, the finding that methionine did not block the covalent binding of the intermediate formed from phenacetin *N*-*O*-sulfate suggests that its reaction with methionine is slow compared with its rate of binding to nucleophilic groups in protein and its rates of conjugation with glutathione and cysteine. Moreover, adenine and guanosine also failed to block the covalent binding of the reactive intermediate. Thus, the pattern of chemical reactivities of the reactive intermediate formed from the *N*-*O*-conjugates of phenacetin toward various nucleophilic substances differs markedly from that of the intermediate formed from the *N*-*O*-conjugates of 2-acetylaminofluorene. This difference may partially account for the fact that 2-acetylaminofluorene is much more carcinogenic than phenacetin, if indeed phenacetin is carcinogenic at all. Presumably the reactive intermediate of phenacetin would be preferentially inactivated by glutathione and protein SH groups, rather than reacting with RNA or DNA, whereas the reactive intermediate of 2-AAF is less rapidly inactivated by glutathione and other amino acids compared with its reaction with nucleic acid.

It is probable that *N*-acetylimidoquinone is also formed by pathways of metabolism of phenacetin other than those mediated by *N*-hydroxyphenacetin. For example, the reactive metabolite of phenacetin that is formed directly by cytochrome P-450 also is readily reduced by ascorbic acid and also forms a glutathione conjugate that lacks an *O*-ethyl group [12, 22]. Because about 50 per cent of the oxygen in the para position of the glutathione conjugate originates from oxygen, it seems plausible that phenacetin is first converted to phenacetin-3,4-epoxide which is de-ethylated to 4,4-dihydroxyacetylimidoquinone and then dehydrates to acetylimidoquinone [12]. On the other hand, the reactive metabolite of acetaminophen formed in liver microsomes does not contain oxygen originating from atmospheric oxygen [12] but is readily reduced by ascorbic acid [22], and thus it probably is formed by *N*-hydroxylation of acetaminophen followed by dehydration to form the acetylimidoquinone. Thus, these studies on the stability and reactions of the reactive intermediate formed from phenacetin *N*-*O*-conjugates may be relevant to other pathways of activation of phenacetin and acetaminophen.

Although the mechanism of phenacetin-induced nephrotoxicities is not known [10, 11], it seems plausible that phenacetin *N*-*O*-glucuronide may play a role since it would be produced primarily in the liver and concentrated in the collecting ducts of the kidney. At this point, its conversion to a reactive intermediate, possibly accelerated by the low pH of urine, could lead to covalent binding and the renal papillary damage associated with phenacetin abusers.

Acknowledgements—We are indebted to Dr. Sidney D. Nelson for mass spectrometric data. G.J.M. was supported by a stipend from the Netherlands Organization for the Advancement of Pure Research (ZWO) and a grant from Hoffmann-La Roche, Inc., Nutley, NJ. His present address is: Department of Pharmacology, State University of Groningen, Groningen, The Netherlands.

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