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GENERATION OF REACTIVE METABOLITES OF *N*-HYDROXY-PHENACETIN BY GLUCURONIDATION AND SULFATION

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Abstract—Several xenobiotics exert their toxic effects in mammals through the formation of reactive metabolites that combine with cellular macromolecules. Thus, *N*-hydroxy-2-acetylaminofluorene becomes covalently bound to various cellular macromolecules after sulfation of the *N*-hydroxy group. A method is presented for the indirect measurement of the rate of formation of the *N*,*O*-sulfate conjugate of this compound, which is too unstable to be measured directly. In this assay 3′,5′-adenosine diphosphate and *p*-nitrophenylsulfate were used as a 3′-phosphoadenosine 5′-phosphosulfate-generating system (PAPS-GS); the release of *p*-nitrophenol (which was measured spectrophotometrically) was used to estimate the sulfation rate of *N*-hydroxy-2-acetylaminofluorene. The PAPS-GS was also used in studying the role of *N*-*O*-sulfate conjugates as intermediates in the formation of reactive metabolites that become covalently bound. Using this method we found that *N*-hydroxy-phenacetin became rapidly covalently bound after sulfation of the *N*-hydroxy group. *N*-hydroxy-2-acetylaminonaphthalene also became covalently bound after sulfation, but the *N*-*O*-sulfate derivatives of *N*-hydroxy-*p*-chloroacetanilide and *N*-hydroxy-acetanilide did not bind covalently, although their rates of sulfation were similar to that of *N*-hydroxy-phenacetin. Glucuronidation of the *N*-hydroxy group of *N*-hydroxy-phenacetin resulted in a glucuronide conjugate that was bound covalently to protein at pH 7.4, but at a slower rate than the *N*-*O*-sulfate conjugate. The *N*-*O*-glucuronides of the other *N*-hydroxy-*N*-arylacetamides investigated did not become covalently bound to protein at pH 7.4. Characteristics of the conjugation of *N*-hydroxy-phenacetin, and of the covalent binding of its conjugates, were determined.

The hepatic carcinogenicity caused by 2-acetylaminofluorene (2-AAF) is thought to be mediated by a series of chemically reactive metabolites [1, 2]. The 2-AAF first undergoes *N*-hydroxylation to form *N*-hydroxy-2-AAF, which in turn is converted either to an *N*-*O*-sulfate conjugate by a sulfotransferase in liver cytosol [3, 4] or to an *N*-*O*-glucuronide by UDP glucuronyltransferase in liver endoplasmic reticulum [5]. Since these conjugates are more chemically reactive than *N*-hydroxy-2-AAF, their formation increases the rate of covalent binding of the toxicant to cellular macromolecules, but the reactivity of the *N*-*O*-glucuronide conjugate is lower than that of the *N*-*O*-sulfate conjugate as measured by their relative rates of covalent binding to macromolecules [5].

Since phenacetin and *p*-chloroacetanilide are also *N*-hydroxylated by hamster liver microsomes [6–8]*, we have studied the effect of sulfation and glucuronidation on the covalent binding of their *N*-hydroxy derivatives to protein. The results indicate that the formation of *N*-*O*-sulfate and *N*-*O*-glucuronide derivatives increases the covalent binding of *N*-hydroxy-phenacetin but does not lead to the covalent binding of the *N*-hydroxy derivatives of *p*-chloroacetanilide and acetanilide.

In the course of this work we developed an easy method for sulfation of the *N*-hydroxy group by a sulfotransferase from rat liver postmicrosomal supernatant, based on the method described by Gregory and Lipmann [9]. In this assay *p*-nitrophenylsulfate is used to convert 3′,5′-adenosine diphosphate to 3′-phosphoadenosine 5′-phosphosulfate (PAPS), a reaction catalyzed presumably by phenolsulfotransferase in the postmicrosomal supernatant of rat liver [10]. The sulfate in PAPS is then transferred to the *N*-hydroxy group and the rate of *p*-nitrophenol release (after donation of the sulfate group) is a measure of the sulfation rate.

* J. A. Hinson and J. R. Mitchell, submitted for publication.

MATERIALS AND METHODS

Chemicals. The *N*-hydroxy-*N*-arylacetamides were synthesized by reduction of the nitro analogue in the presence of zinc to the hydroxylamine, followed by acetylation with acetylchloride, as previously described [7, 11, 12]. For the preparation of ^{14}C-labeled *N*-hydroxy-*N*-arylacetamides, [1-^{14}C]acetylchloride (New England Nuclear, Boston, Mass., U.S.A.) was used. Specific radioactivities of the compounds were about 4 mCi/m-mole. Identity of the *N*-hydroxy-*N*-arylacetamides was confirmed by electron impact mass spectrometry and their purity was established by thin-layer chromatography on Silica gel plates with ether as solvent. 3′,5′-Adenosine diphosphate (PAP, UDP glucuronate, Triton X-100 and *p*-nitrophenylsulfate were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. The other organic sulfates used in this study were purchased from ICN Pharmaceuticals, Cleveland, Ohio, U.S.A. Bovine serum albumin (fraction V from bovine plasma) was obtained from Armour Pharmaceuticals, Chicago, Ill., U.S.A.

NADH and phosphoenolpyruvate were obtained from CalBiochem, Los Angeles, Calif., U.S.A., and the enzymes for the NADH-linked UDP glucuronyltransferase assay [13] were purchased from Boehringer, Mannheim, Germany. [D-Glucuronic acid-^{14}C(U)]-UDP glucuronic acid was obtained from New England Nuclear, Boston, Mass., U.S.A.

Rats and enzyme preparations. Adult male Sprague–Dawley rats (Charles River CDS), 200–300 g, had free access to food and water. The animals were decapitated and the livers were 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. The homogenates were centrifuged at 9000 *g* for 30 min to remove nuclei, lysosomes and mitochondria, and the supernatants were then centrifuged at 100,000 *g* for 60 min to sediment the microsomes. The microsomes were washed once by resuspending them in buffered KCl and centrifuging them at 100,000 *g* for 60 min. The pellets were resuspended in the buffered KCl to give a protein concentration of about 10 mg/ml. Unless otherwise indicated, the microsomes were treated with Triton X-100 described previously [14].

The postmicrosomal supernatant was chromatographed over Sephadex G-25 (45 × 2.5 cm column) with 50 mM sodium phosphate buffer, pH 7.4, as eluent, to remove glutathione or other low molecular weight substances which may interfere with the assay of covalent binding. The chromatographed postmicrosomal supernatant was used as the source of sulfotransferase and contained about 25 mg protein/ml.

Protein determination. Protein was determined according to the method of Lowry *et al.* [15] using bovine serum albumin as standard.

Sulfotransferase assay. The incubation mixture in a 1-cm light-path cuvette contained (final concentrations) 100 mM Tris–HCl, pH 8.0; 10 mM *p*-nitrophenyl-sulfate; 20μM 3′,5′-adenosine diphosphate; and 0.5 mM *N*-hydroxy-2-AAF (or one of the other substrates) added as an ethanol–water (1:1) solution such that 5% ethanol (v/v) was present in the incubation medium. After addition of the postmicrosomal supernatant (about 600 μg protein/ml of incubation medium) the cuvette was placed in a spectrophotometer and the rate of increase in absorbancy at 405 nm was recorded at 31°. The rate of formation of *p*-nitrophenol in control incubation mixtures, which did not contain the acceptor substrate, was subtracted from the values of the experimental mixtures. The rate of sulfation was calculated from the amount of *p*-nitrophenol released using a molar extinction coefficient of 17,500 M^{-1} cm^{-1} (at pH 8.0). The difference between duplicates was always less than 10 per cent.

UDP glucuronyltransferase assay. The UDP glucuronyltransferase assay was performed as previously described [13] using the NADH-linked assay. Substrates were added in an ethanol–water (1:1) solution. The substrate concentration was 0.3 mM in the incubation, and UDPGA was present at 2.0 mM. Triton X-100-activated microsomes [14] were usually used as a source of UDP glucuronyltransferase.

Covalent binding assay. The incubation medium for assay of covalent binding of the *N*-hydroxy-arylacetamides contained (final concentrations): 5 mM $MgCl_2$; 20 mM sodium phosphate buffer, pH 7.4; 75 mM KCl; 5 mM Tris–HCl buffer, pH 7.4; and 0.5 mM of the ^{14}C-labeled *N*-hydroxy-arylacetamide. When the effect of glucuronidation on covalent binding of the *N*-hydroxy-arylacetamide was studied, the incubation medium also contained 2.0 mM UDP glucuronate and about 2.0 mg/ml of microsomal protein (treated with Triton X-100 unless otherwise indicated); in some experiments postmicrosomal supernatant (7.5 mg protein/ml) was also included. When the effect of sulfation on covalent binding was studied, the incubation mixtures contained 10 mM *p*-nitrophenylsulfate, 20 μM PAP and about 7.5 mg/ml of postmicrosomal supernatant protein.

In the experiment of Table 2, covalent binding was measured under the same incubation conditions as the sulfotransferase assay, except that radiolabeled substrates were used and bovine serum albumin (to 6 mg/ml) was added to provide binding sites for covalent binding. In the experiment reported in Table 4, the concentration of postmicrosomal supernatant protein was higher than it was in the sulfotransferase assay (see footnote to Table 4).

The mixtures were incubated at 37° usually for 5–10 min. The reactions were terminated by the addition of methanol (final concentration 80%) and the denatured protein was washed twice with 40% (v/v) methanol–water and five times with ethyl ether–ethanol (60:20) or until the radioactivity of the wash fluid was no more than 30 cpm/2 ml above background. The protein from 1 ml of incubation medium was suspended in 0.3 ml of 1 N NaOH and dissolved by warming the tubes. Radioactivity in the solution was determined in a liquid scintillation counter. The amount of metabolite covalently bound to protein was calculated from the specific radioactivity of the *N*-hydroxy-*N*-arylacetamides. All experiments were run in duplicate, and repeated at least once, to make sure that the results were reproducible. The differences between duplicates were always less than 10 per cent.

As described above, covalent binding is assumed to have taken place when radioactivity derived from the substrate cannot be removed from protein by extensive washing with 80% methanol and ethyl ether–ethanol. Jollow *et al.* [16] have shown that covalent binding of acetaminophen to liver protein as determined by this method indeed represented covalent binding of a reactive metabolite to amino acids in protein.

Thin-layer chromatography of the N-*hydroxy*-N-*arylacetamides and their glucuronides.* The substrates were separated from their glucuronides by thin-layer chromatography at room temperature on DEAE cellulose plates (Avicel F; 250 μm) with *n*-propanol–0.4 M ammonium hydroxide (80:20) as the developing solvent. The R_f value of *N*-hydroxyphenacetin was 0.85, that of its glucuronide conjugate 0.50. Similar values were found for *N*-hydroxy-*p*-chloroacetanilide and its glucuronide conjugate. Plates were scanned for ^{14}C radioactivity by a Packard 7301 Radiochromatogram scanner. The radioactive spots were scraped from the plate and the compounds were extracted in 80% (v/v) methanol–water overnight at room temperature. Recovery of ^{14}C radioactivity applied to the plate was between 85 and 95 per cent.

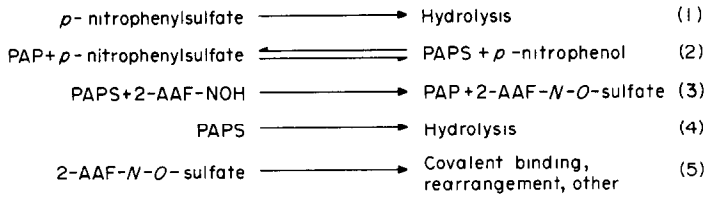

Fig. 1. Reactions occurring under the conditions of the sulfotransferase assay.

RESULTS AND DISCUSSION

Rapid assay for sulfation of the N-*hydroxy group.* We used the assay of phenolsulfotransferase as described by Gregory and Lipmann [9] for measurement of the sulfation rate of the *N*-hydroxy-*N*-arylacetamides. The reactions of this assay are given in Fig. 1. In the presence of an acceptor substrate for the sulfate group, in this case *N*-hydroxy-2-AAF, there is a rapid release of *p*-nitrophenol as the sulfate group is transferred from *p*-nitrophenylsulfate to the *N*-hydroxy group of the substrate (Fig. 2). In the absence of *N*-hydroxy-2-AAF, there is an initial rapid rate of increase in the yellow color of the sulfotransferase assay medium (Fig. 2) caused by the accumulation of PAPS (Fig. 1, reaction 2); after about 2 min this rate becomes slower and constant. The addition of *N*-hydroxy-2-AAF greatly increased the rate of appearance of the yellow color of *p*-nitrophenol (Fig. 2; Fig. 1, reactions 2 and 3). Since a similar increase did not occur when PAP was omitted from the incubation (Fig. 1, Table 1), the sulfate group of *p*-nitrophenylsulfate is not transferred directly to *N*-hydroxy-2-AAF. Similarly, when the postmicrosomal supernatant, *p*-nitrophenylsulfate or other components were deleted, little or no *p*-nitrophenol was formed (see Table 1). Thus, the rate of hydrolysis of *p*-nitrophenylsulfate by sulfatases was small. For determination of the sulfation rate of a substrate we used routinely the period of 3–6 min after the start of the incubation since by then the rate of increase of the yellow color had stabilized. Within any series of measurements the variation between duplicates was always less than 10 per cent, usually about 5 per cent.

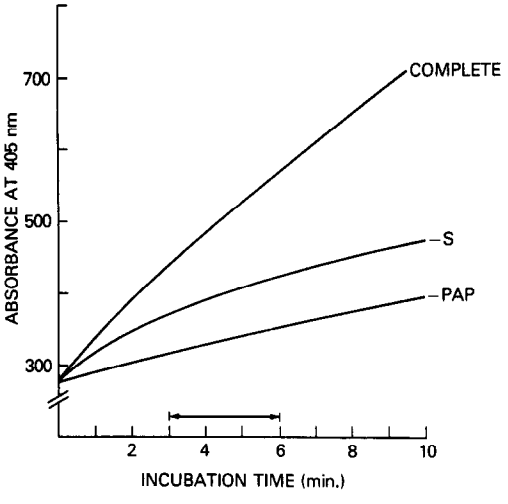

Fig. 2. Effect of *N*-hydroxy-2-AAF on the formation of free *p*-nitrophenol from *p*-nitrophenylsulfate, catalyzed by rat liver postmicrosomal supernatant. The assay medium was as described in Materials and Methods. Absorbance at 405 nm was measured continuously. When PAP or *N*-hydroxy-2-AAF (S) was deleted, only a slow increase in the yellow color occurred. The bar indicates the period in which measurement of reaction rate was made.

The sulfation rate seemed not to be limited by the rate of PAPS generation, because phenol was sulfated at an 8- to 10-fold higher rate when it was used as the acceptor (however, phenol sulfation occurred without PAP being added; see below). The reaction rate was linear with postmicrosomal protein concentration up to 1200 μg/ml and was about 2.7 nmoles *p*-nitrophenol formed/min/mg of postmicrosomal supernatant protein (at 31°). These results suggest that the *N*-*O*-sulfate of *N*-hydroxy-2-AAF had been formed, but the isolation of this metabolite is impossible because of its instability. The finding that the sulfation of *N*-hydroxy-2-AAF depended on the presence of PAP was surprising because sulfation of phenol in our system was completely independent of PAP even though this reaction occurs more rapidly than does sulfation of *N*-hydroxy-2-AAF. However, the results of Gregory and Lipmann [9] suggest that impure enzyme preparations may be independent of added PAP, presumably because they contain trace amounts of PAP.

Evidence that an *N*-*O*-sulfate conjugate was formed in the incubation mixtures was obtained by showing that the covalent binding of *N*-hydroxy-2-AAF (Fig. 1, reaction 5) increased dramatically during incuba-

Table 1. Effect of deletion of various cofactors from the sulfotransferase assay medium on increase of yellow color (extinction at 405 nm)*

Conditions	ΔE_{405}/5 min
Complete system	195
Minus S	75
Minus NPS	30
Minus PAP	70
Minus enzyme	5
Minus PAP and S	20
Minus PAP and pNPS	20
Minus pNPS and S	15
Minus S + phenol (0.5 mM)	1250†

* S = *N*-hydroxy-2-AAF; pNPS = *p*-nitrophenylsulfate; enzyme = postmicrosomal supernatant. Incubation was as indicated in Materials and Methods.

† This velocity is based on the initial rate.

Table 2. Effect of sulfation on covalent binding of *N*-hydroxy-2-AAF*

Concentration of postmicrosomal protein (mg/ml)	PAP	pNPS	*N*-hydroxy-2-AAF (nmoles bound/ml)
0.6	+	−	0.3
	−	+	2.1
	+	+	7.4
7.5	+	−	0.3
	−	+	26.6
	+	+	36.5

* The incubation medium (1 ml) contained either 0.05 or 0.3 ml of the postmicrosomal supernatant (Sephadex G-25 treated) to give the indicated protein concentrations, 500 nmoles of *N*-hydroxy-2-AAF substrate/ml and 6 mg/ml of bovine serum albumin. In separate tubes either *p*-nitrophenylsulfate (pNPS; 10 mM) or PAP (20 μM) was deleted from the incubation medium. The incubation was for 10 min at 31°.

tion with *p*-nitrophenylsulfate and PAP (Table 2). DeBaun *et al.* [4] have extensively studied this effect of sulfation on covalent binding of *N*-hydroxy-2-AAF. The dependence of the reaction on PAP decreased when more of the postmicrosomal supernatant was added, suggesting that PAP was not completely removed by the Sephadex G-25 chromatography.

We have used this system to measure sulfation rate of several *N*-hydroxy-*N*-arylacetamides and to synthesize the *N*-*O*-sulfates of these compounds for covalent binding studies.

Effect of sulfation on covalent binding of N-*hydroxy*-N-*arylacetamides.* All of the *N*-hydroxy-arylacetamides tested can form sulfate derivatives, at rates comparable to that of *N*-hydroxy-2-AAF (Table 3). Sulfation of all but one of the substrates was completely dependent on the presence of PAP; the sulfation of *N*-hydroxy-*p*-chloroacetanilide was only about 70 per cent dependent on PAP.

In accordance with the finding of Hinson and Mitchell,* *N*-hydroxy-phenacetin did not bind covalently to protein at pH 7.4 in the absence of *p*-nitrophenylsulfate. In the presence of *p*-nitrophenylsulfate and PAP, however, considerable amounts of *N*-hydroxy-phenacetin became bound when it was incubated with a postmicrosomal supernatant fraction (Table 4), as was also the case with *N*-hydroxy-2-AAF. Covalent binding of *N*-hydroxy-2-acetylaminoaphthalene was increased to a lesser extent after *N*-*O*-sulfation in agreement with the results of DeBaun *et al.* [4]. Sulfation of *N*-hydroxyarylacetamides does not always result in appreciable covalent binding; there was little or no increase in the covalent binding of *N*-hydroxy-acetanilide and *N*-hydroxy-*p*-chloroacetanilide after addition of PAP and *p*-nitrophenylsulfate.

Several other phenylsulfates were tested as possible donors of the sulfate group to PAP. Only 4-methyl-umbelliferylsulfate increased the covalent binding of *N*-hydroxy-phenacetin, but only to about 20 per cent of the binding measured with *p*-nitro-phenylsulfate as donor; phenolphthalein-disulfate, 2-hydroxy-5-nitro-phenylsulfate and 6-benzoyl-2-naphthylsulfate had no effect (all sulfates were tested at 1 mM final concentration). At a low concentration of postmicrosomal supernatant (0.6 mg protein/ml), covalent binding of *N*-hydroxy-phenacetin was nearly completely dependent on the presence of PAP; omission of PAP from the incubation mixture decreased the covalent binding by about 85 per cent. However, at a higher concentration of postmicrosomal supernatant (7.5 mg protein/ml), the omission of PAP decreased this binding only 30 per cent (Fig. 3), an effect similar to that shown in Table 2 with *N*-hydroxy-2-AAF.

* Submitted for publication.

Table 3. Conjugation rates of some *N*-hydroxy-*N*-arylacetamides†

Substrate	Sulfation rate (nmoles/min/mg postmicrosomal supernatant protein)	Glucuronidation rate (nmoles/min/mg microsomal protein)
N-hydroxy-phenacetin	2.6	12
N-hydroxy-acetanilide	1.6	5
N-hydroxy-*p*-chloroacetanilide	2.4	17
N-hydroxy-2-AAF	2.7	10
N-hydroxy-2-acetylaminonaphthalene	2.0	19
(4-Bromophenol)		(40)

† Sulfation rates were measured with *p*-nitrophenylsulfate as sulfate donor in the presence of PAP, and postmicrosomal supernatant as enzyme preparation (about 600 μg/ml of protein in the incubation) at pH 8.0. The acceptor substrates were present at 0.5 mM. Rates were corrected for the increase in yellow color in the absence of the acceptor substrate. The glucuronidation rates were measured with Triton X-100-activated microsomes (400 μg microsomal protein/ml) at pH 7.4. The substrate concentration was 0.03 mM.

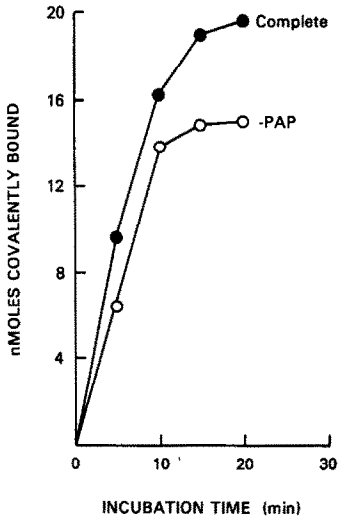

Fig. 3. Time course of covalent binding of *N*-hydroxy-phenacetin. The concentration of *p*-nitrophenylsulfate was 10 mM and that of PAP was 0.02 mM. The incubation was at pH 7.4. The amount of nmoles bound to protein/ml of incubation medium (containing 7.5 mg/ml of postmicrosomal supernatant protein) is given.

Characteristics of the effect of sulfation on covalent binding of N-*hydroxy-phenacetin.* Several facts indicate that the increase in covalent binding of *N*-hydroxy-phenacetin in the presence of PAP and *p*-nitrophenyl-sulfate is due to the enzyme sulfotransferase: (1) the increase was dependent on the presence of both *p*-nitrophenylsulfate and PAP; (2) at 0°, *p*-nitrophenylsulfate and PAP did not increase covalent binding of *N*-hydroxy-phenacetin; and (3) a boiled postmicrosomal supernatant (2 min at 100°) did not increase covalent binding, nor did an equivalent amount of bovine serum albumin in the presence of both *p*-nitrophenyl-sulfate and PAP. Therefore, a direct chemical transfer of sulfate from *p*-nitrophenyl-sulfate to PAP or *N*-hydroxy-phenacetin seems unlikely.

The rate of covalent binding of *N*-hydroxy-phenacetin was nearly linear during the first 10 min. However, after 10 min of incubation at 37°, the reaction rate decreased rapidly (see Fig. 3). During the first 5 min the rates of both covalent binding and sulfation showed a similar dependence on *N*-hydroxy-phenacetin concentration (Fig. 4): a K_m of 0.5 mM was found for covalent binding measured at pH 7.4, and of 0.25 mM for sulfation (measured at different conditions). Because we were unable to measure sulfation rates at concentrations of postmicrosomal supernatant protein over 1 mg/ml (due to a high absorbancy of the enzyme preparation at 405 nm), we could not determine whether the sulfation rate was linear with enzyme concentration up to 7.5 mg protein/ml and thus could not determine the relationship between the V_{max} value for sulfation and covalent binding from these data. In another experiment in which the sulfation rate and the covalent binding were measured under exactly the same conditions, we found that at least 65 per cent of the amount of *N*-hydroxy-phenacetin and *N*-hydroxy-2-AAF that became sulfated, became covalently bound to protein.

The increased rate in covalent binding of *N*-hydroxy-phenacetin by the PAPS-generating system was strongly inhibited by *p*-nitrophenol (80 per cent inhibition at 0.1 mM), presumably because *p*-nitrophenol competes with *N*-hydroxy-phenacetin either for PAPS or for the sulfotransferase (see DeBaun *et al.* [4]). Glutathione also markedly inhibited covalent binding (90 per cent at 0.1 mM) not only by competing with protein for the reactive metabolite but also by inhibiting the rate of sulfation (30 per cent inhibition at 1.0 mM). Finally, methionine (up to 20 mM) did not affect the degree of covalent binding to protein of the sulfate conjugate of *N*-hydroxy-phenacetin, whereas it reduced this covalent binding of the *N*-*O*-sulfate of *N*-hydroxy-2-AAF to less than 7 per cent (at 20 mM methionine) of that in the absence of methionine. For *N*-hydroxy-2-AAF this is to be expected, since methionine and protein are competing for trapping of the reactive metabolite[4]. The fact that methionine does not inhibit covalent binding to protein of the *N*-hydroxy-phenacetin *N*-*O*-sulfate conjugate suggests, however, that the mechanism of covalent binding may be different for the sulfate conjugates of *N*-hydroxy-2-AAF and hydroxy-phenacetin.

The present results clearly show that *N*-hydroxy-phenacetin can become covalently bound when its *N*-hydroxy group is sulfated. Since *N*-*O*-sulfates of this type usually are very unstable in aqueous media [5, 17, 18] it presumably would be impossible to isolate this conjugate from the incubation medium. However, substituents in the phenyl ring determine the chemical reactivity of the *N*-*O*-sulfate conjugates, as measured by covalent binding. Thus, the presence of a chlorine atom instead of the ethoxy group in the *para*-position alters the chemical reactivity of the *N*-*O*-sulfate conjugate to such an extent that it no longer can act as an arylating metabolite. However, since the ^{14}C label is in the acetyl group, only binding of those metabolites in which this group is conserved

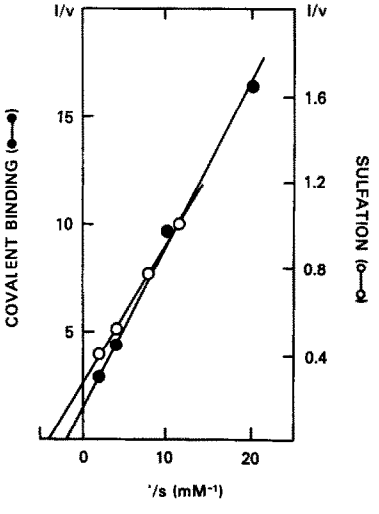

Fig. 4. Lineweaver–Burk plot of covalent binding (●——●) and sulfation (○——○) of *N*-hydroxy-phenacetin. Sulfation was measured at pH 8.0 at 600 μg/ml of postmicrosomal supernatant protein at 31°. Covalent binding was measured at pH 7.4 at 7.5 mg/ml of postmicrosomal supernatant protein at 37°. Incubation for covalent binding was for 5 min. *V* is expressed as nmoles/min/mg of protein.

Table 4. Effect of sulfation on covalent binding of some *N*-hydroxy-*N*-arylacetamides*

Substrate	Amount covently bound (nmoles/ml)	
	With pNPS	Without pNPS
N-hydroxy-phenacetin	25.0	0.2
N-hydroxy-acetanilide	0.2	0.2
N-hydroxy-*p*-chloroacetanilide	0.4	0.2
N-hydroxy-2-AAF	21.8	0.6
N-hydroxy-2-acetylaminonaphthalene	1.9	0.2

* The incubation medium contained 7.5 mg/ml of postmicrosomal supernatant protein and 500 nmoles substrate/ml. The incubation was for 10 min at pH 8.0. PAP was present at 20 μM. pNPS = *p*-nitrophenylsulfate (10 mM). The total amount of nmoles bound to protein/ml of incubation medium is given.

was measured. The lack of covalent binding of *N*-hydroxy-acetanilide and *N*-hydroxy-*p*-chloroacetanilide in the presence of the PAPS-generating system may indicate that either their *N*-*O*-sulfate conjugates are unusually stable, or rearrange preferentially to a non-reactive metabolite, or lose their *N*-acetyl group in the process of covalent binding to the protein.

The chemical mechanism involved in the covalent binding of the *N*-*O*-sulfate of *N*-hydroxy-phenacetin is unknown, but it may be similar to the mechanism postulated by Miller and Miller [1] for the covalent binding of the *N*-*O*-sulfate of *N*-hydroxy-2-AAF. Another possible mechanism would involve the spontaneous formation of the arylating species acetimidoquinone, which has been shown by Calder *et al.* [19] to be spontaneously formed from *N*-hydroxy-phenacetin in aqueous acid media.

Effect of glucuronidation on covalent binding of N-*hydroxy-phenacetin.* The *N*-hydroxy-*N*-arylacetamides used in the present study were glucuronidated *in vitro* by rat liver microsomal UDP glucuronyltransferase activated with Triton X-100. Table 3 shows that the glucuronidation rates were comparable, but lower than that of 4-bromophenol used as a reference [13].

The effect of glucuronidation on covalent binding of *N*-hydroxy-phenacetin to rat liver protein is shown in Table 5. After incubation with Triton X-100-activated microsomes, *N*-hydroxy-phenacetin became covalently bound only when UDPGA was present. Without Triton X-100 activation the activity of the enzymes *in vitro* was much lower and only little covalent binding took place. The presence of postmicrosomal supernatant had no effect upon glucuronidation, but stimulated covalent binding considerably, presumably by presenting more sites for covalent binding. Thin-layer chromatography (t.l.c.) of a sample of the incubation medium (after deproteinization with methanol) revealed the presence of the glucuronide of *N*-hydroxy-phenacetin, confirming the results of the NADH-linked UDP glucuronyltransferase assay. Experiments in which we used UDP glucuronate that was labeled in the glucuronate group with ^{14}C, to glucuronidate *N*-hydroxy-phenacetin, confirmed the identity of the glucuronide on the t.l.c. plate. When *N*-hydroxy-2-AAF was glucuronidated under identical conditions no covalent binding took place; thus, the *N*-*O*-glucuronide conjugate of *N*-hydroxy-phenacetin is more reactive than that of *N*-hydroxy-2-AAF, which binds to a considerable extent to protein only under alkaline conditions [20]. When *N*-hydroxy-acetanilide, *N*-hydroxy-*p*-chloroacetanilide or *N*-hydroxy-2-AAF was incubated with Triton X-100-treated microsomes, no covalent binding occurred either in the presence or absence of UDP glucuronate. Since thin-layer chromatography showed that the *N*-*O*-glucuronide of *N*-hydroxy-*p*-chloroacetanilide was formed even more rapidly than the glucuronide of *N*-hydroxy-phenacetin, the lack of covalent binding suggests that the *N*-*O*-glucuronides of these substances have low chemical reactivity.

A time course of glucuronidation and covalent

Table 5. Glucuronidation of *N*-hydroxy-phenacetin and covalent binding of the conjugate*

Incubation medium	Amount covalently bound (nmoles/ml)		Percent of *N*-hydroxy-phenacetin glucuronidated	
	After 30 min	After 60 min	After 30 min	After 60 min
Complete	6.3	18.0	74	84
Without UDP glucuronate	0.5	0.5	0	0
Without postmicrosomal supernatant	3.3	7.8	73	86
Without Triton X-100	0.7	2.6	23	40

* The concentration of *N*-hydroxy-phenacetin was 500 nmoles/ml. Microsomal protein concentration was 2.4 mg/ml; the microsomal preparation was activated with Triton X-100 unless indicated otherwise. Postmicrosomal supernatant was present at a concentration of 6.9 mg/ml, unless otherwise indicated. The total amount of nmoles bound to protein/ml of incubation medium is given.

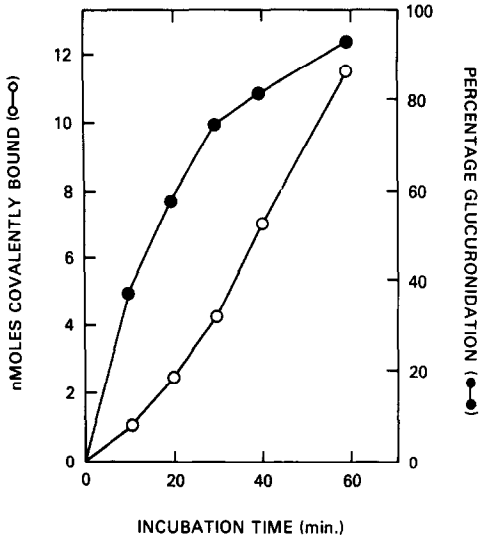

Fig. 5. Glucuronidation and covalent binding of N-hydroxy-phenacetin by rat liver microsomes. The microsomal protein concentration was 2.3 mg/ml (Triton X-100 activated), and 6.9 mg/ml of postmicrosomal supernatant protein was present. The percentage of glucuronidation was estimated by separation of N-hydroxy-phenacetin and its glucuronide on a t.l.c. plate and subsequent elution and measurement of the radioactivity in the spots. Covalent binding is expressed as nmoles bound/ml of incubation medium. The concentration of N-hydroxy-phenacetin was 500 nmoles/ml.

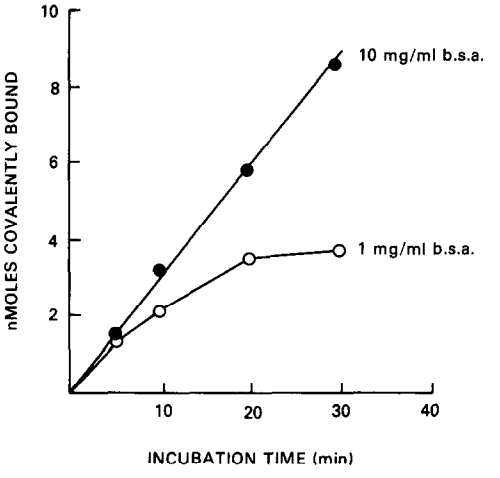

Fig. 6. Binding of the N-O-glucuronide conjugate of N-hydroxy-phenacetin to bovine serum albumin. A solution of the N-O-glucuronide conjugate of N-hydroxy-phenacetin (about 0.4 mM; see legend to Fig. 3) was incubated with bovine serum albumin (b.s.a.) at 37° at pH 7.4. Covalent binding is expressed as nmoles bound/ml of incubation medium.

binding (Fig. 5) showed that the rate of covalent binding shows a lag phase as compared with the rate of glucuronidation. First the glucuronide conjugate of N-hydroxy-phenacetin accumulated and covalent binding accelerated as the concentration of the glucuronide increased to a steady state value. About 0.03 to 0.04 nmole/min/mg of protein was bound during the linear part of the curve (see Fig. 5). By contrast there was no lag phase in the binding of the N-O-sulfate conjugate, which seems to bind almost as rapidly as it is formed.

In order to show that the covalent binding of the glucuronide of N-hydroxy-phenacetin is independent of the presence of microsomal protein, once it is formed, we synthesized the glucuronide conjugate by incubation of N-hydroxy-phenacetin with microsomes and UDP glucuronate, and removed all protein by precipitation with methanol. About 80 per cent of the N-hydroxy-phenacetin initially present was converted to the glucuronide after incubating the mixture for 1 hr at 37°. The methanol–water layer was evaporated with a stream of nitrogen at room temperature and the residue was dissolved in the original volume of water. Addition of bovine serum albumin to this solution and incubating the mixture at 37° resulted in the binding of the metabolite covalently to albumin (Fig. 6). The rate of binding to albumin was 0.03 nmole/min/mg of protein. At a lower albumin concentration the rate of covalent binding decreased during the incubation, possibly due to a decrease in available binding sites. When UDP glucuronate was omitted from the incubation mixture, no N-O-glucuronide was formed and no covalent binding occurred.

Glucuronide conjugates of the N-hydroxy group are usually less stable than phenolic hydroxyl group conjugates [5, 21]. Thus, the glucuronide of N-hydroxy-phenacetin was unstable at 100°; when it was heated for 2 min at pH 7.4 in a boiling water bath the glucuronide peak disappeared from the thin-layer chromatogram and a rapid moving peak with an R_f of about 0.9 appeared. Since the glucuronide of N-hydroxy-p-chloroacetanilide was not hydrolyzed under these conditions, it presumably is more stable than its phenacetin analogue. Moreover, when a solution of the glucuronide of N-hydroxy-phenacetin was heated for 2 min at 100° in the presence of protein, the amount covently bound to protein was markedly increased (Table 6). But under these conditions, the glucuronide of N-hydroxy-p-chloroacetanilide was still not covalently bound. Therefore, the stability of the glucuronide of N-hydroxy-p-chloroacetanilide may prevent its becoming bound covalently, whereas the instability of the glucuronide conjugate of N-hydroxy-phenacetin favors covalent binding. In conclusion, the N-O-glucuronide of N-hydroxy-phenacetin seems to be unusually reactive at pH 7.4 as measured by its covalent binding to protein.

General comments. These results suggest that p-nitrophenylsulfate and PAP may be used to measure sulfation rates of N-hydroxy-arylacetamides. DeBaun *et al.* [4] have used methionine to capture the reactive N-O-sulfate conjugate of N-hydroxy-2-AAF and have based their sulfotransferase assay on the production of the methionine-bound AAF. However, other degradation products of this N-O-sulfate are possible and the actual sulfation rate may be much higher than the rate of binding to methionine. Moreover, N-O-sulfate conjugates of other N-hydroxy-arylacetamides might not bind at all to methionine (as seems the case with the N-O-sulfate conjugate of N-hydroxy-phenacetin) and thus might seem to be

Table 6. Effect of temperature on covalent binding of *N*-*O*-glucuronides*

Glucuronide	Amount covalently bound (nmoles/ml)	
	Without heating	After heating at 100°
N-hydroxy-phenacetin glucuronide	2.4	18.4
N-hydroxy-*p*-chloroacetanilide glucuronide	0.0	0.0

* After a normal covalent binding incubation for 60 min at 37°, the reaction was stopped with methanol in the tubes "without heating"; the tubes "after heating at 100°" were put in a boiling water bath for 2 min, and thereafter methanol was added on ice. The microsomal protein concentration was 2.0 mg/ml and no postmicrosomal protein was present during the incubation. The amount of nmoles bound to protein/ml of incubation medium is given.

not sulfated according to the covalent binding assay. We feel that the present assay offers a tool to measure sulfation rates more directly than the methionine-binding assay. Moreover, with the present method the rate of sulfation and the rate of covalent binding can be evaluated separately. Further, *p*-nitrophenylsulfate and PAP may be used to synthesize the *N*-*O*-sulfate conjugates of *N*-hydroxy-arylacetamides *in situ* for covalent binding studies, avoiding the need of PAPS, which is not commercially available in the unlabeled form and the synthesis of which is lengthy.

The present results clearly show that the *N*-*O*-sulfate of *N*-hydroxy-phenacetin binds covalently to protein as soon as it is synthesized. Since phenacetin is *N*-hydroxylated by microsomal enzymes [6–8],* it seems likely that the sulfation and glucuronidation pathways provide a reactive intermediate for covalent binding. At the present time, however, it is not clear whether either reaction plays a role in causing the toxic effects of high doses of phenacetin *in vivo*.

Although glucuronidation seems less important for covalent binding in liver, it may be important in kidney and bladder, because many glucuronides are highly concentrated in urine. The fact that the degree of latency of UDP glucuronyl-transferase *in vivo* is unknown [22] makes it difficult to assess its importance *in vivo* relative to sulfation. A more detailed study on pharmacokinetics and metabolism of all phenacetin metabolites *in vivo* may answer the question whether the *N*-hydroxylated metabolites are involved in its toxic effects.

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