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SHORT COMMUNICATIONS

N-hydroxylation of *p*-chloroacetanilide in hamsters

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The *N*-hydroxylation *in vivo* and *in vitro* of polycyclic *N*-acetylarylamines such as 2-acetylaminofluorene has been well established [1, 2]. The role of *N*-hydroxylation in the metabolism of monocyclic *N*-acetylarylamines, however, is uncertain. For example, Poirier *et al.* [3] gave acetanilide and its *p*-vinyl, *p*-fluoro and *p*-ethoxy derivatives to dogs but were unable to detect *N*-hydroxy metabolites in urine even though they found that 2-acetylaminofluorene was converted to *N*-hydroxy-2-acetylaminofluorene in a parallel experiment. Similarly, Kiese and Lenk [4] were unable to detect *N*-hydroxy-*p*-chloroacetanilide in rabbit urine after administration of *p*-chloroacetanilide, and Weisburger, Grantham *et al.* [2, 5] could not detect *N*-hydroxyacetanilide in rat urine after administration of acetanilide. On the other hand, Troll, Belman *et al.* [6, 7] gave acetanilide and phenacetin (*p*-ethoxyacetanilide) to humans and reported the occurrence of *N*-hydroxy metabolites of these substances in the urine. The interpretation of these results, however, has been questioned [2].

We previously reported that acetaminophen (*p*-hydroxyacetanilide) is metabolically activated *in vivo* and *in vitro* by a cytochrome P-450-dependent drug-metabolizing enzyme to a chemically reactive arylating agent [8–11]. This toxic arylating metabolite was postulated to be the acetimidoquinone of acetaminophen which is formed by initial *N*-hydroxylation followed by a spontaneous dehydration [12]. This hypothesis, however, has suffered from lack of evidence that monocyclic *N*-acetylarylamines are *N*-hydroxylated. Recently, we were able to demonstrate that *p*-chloroacetanilide can be *N*-hydroxylated *in vitro* [13]. This compound was used as a model substrate because its *N*-hydroxy derivative is stable and could be synthesized. We have now extended this work to show that this monocyclic *N*-acetylarylamine is *N*-hydroxylated *in vivo*.

N-hydroxy-*p*-chloroacetanilide was synthesized by reduction of *p*-chloronitrobenzene followed by acetylation with acetyl chloride as previously described [13, 14]. The identity of the synthesized material was established by electron impact mass spectrometry, chemical ionization mass spectrometry and nuclear magnetic resonance studies [13]. Carbonyl-^{14}C-labeled *N*-hydroxy-*p*-chloroacetanilide and carbonyl-^{14}C-labeled-*p*-chloroacetanilide were synthesized by acetylation of *p*-chlorophenylhydroxylamine and *p*-chloroaniline, respectively, with [1-^{14}C]acetyl chloride (30·2 mCi/m-mole, New England Nuclear, Boston, Mass.) as previously described [13, 14]. Radiochemical purity was greater than 99·5 per cent, as shown by thin-layer chromatography on Silica gel with the four solvent systems listed in Table 1. All other chemicals were of the purest grade commercially available.

Male golden Syrian hamsters (60 g) were obtained from the Animal Production Center, NIH, and maintained on Purina Lab Chow and water *ad lib.* The hamsters received 3-methylcholanthrene (20 mg/kg, i.p. in sunflower oil) daily for 3 days. Twenty-four hr later, the animals received either [^{14}C]*p*-chloroacetanilide (10 mg/kg, 0·3 μCi/μmole, i.p.) dissolved in 10% Tween 80, or [^{14}C]*N*-hydroxy-*p*-chloroacetanilide (3·3 mg/kg, 0·03 μCi/μmole, i.p.) dissolved in saline. The animals were placed in individual metabolic cages and the urine was collected over dry ice for 24 and 48 hr. Four animals were used in each experiment.

An aliquot of urine from each animal was extracted three times with an equal volume of ether and the ether extracts were combined. The ether fraction contained the nonconjugated metabolites. Another aliquot of urine from each animal was made 0·1 M with respect to sodium phosphate, pH 7·4, and bacterial β-glucuronidase (type I, Sigma Chemical Co., St. Louis, Mo.) was added to a concentration of 1 mg/ml. The mixtures were incubated at 37° for 1·5 hr, and additional β-glucuronidase was added to increase the enzyme concentration to 2 mg protein/ml. The incubations were continued for another 1·5 hr. To facilitate the recovery of the metabolite of *p*-chloroacetanilide, nonradioactive *N*-hydroxy-*p*-chloroacetanilide (250 μg) was then added to each incubation flask. Without this addition, recoveries varied too greatly between duplicates. The incubation mixture was then extracted three times with an equal volume of ether and the ether extracts were combined. The ether extracts, which contained the glucuronide metabolite fraction, were dried under a stream of nitrogen. Further incubation of the aqueous phases with β-glucuronidase failed to liberate significant amounts of radiolabeled material. Total radioactivity in the urine and in each fraction was determined by liquid scintillation spectrometry.

The components that were released after incubation with β-glucuronidase were separated by applying each fraction to a 250-μm thick Silica gel thin-layer chromatographic plate (Analtech, Newark, Del.) and developing the thin-layer plate with ether–heptane (75:25). The radioactive metabolites were located by a strip scanner (Packard model 385 Recording Ratemeter). The regions of Silica gel corresponding to each metabolite were scraped from the plates and the metabolites extracted into methanol. The radioactivity in each metabolite fraction was determined by liquid scintillation spectrometry. The solvent systems used to resolve the *N*-hydroxy-*p*-chloroacetanilide are listed in Table 1.

For determination of the specific activity of the [^{14}C]*N*-hydroxy-*p*-chloroacetanilide metabolite, the radioactivity was measured by liquid scintillation spectrometry. The *N*-hydroxy-*p*-chloroacetanilide concentration was then measured by addition of 5 ml of a methanolic solution of ferric chloride (0·2%) and determination of the optical density of the ferric chelate at 530 nm. The amount of *N*-hydroxy-*p*-chloroacetanilide in the sample was calculated from the molar extinction coefficient (1·44 mM^{-1} cm^{-1}) of authentic *N*-hydroxy-*p*-chloroacetanilide determined under the same conditions.

To determine if *N*-hydroxy-*p*-chloroacetanilide could be recovered as a urinary metabolite, four hamsters pretreated with 3-methylcholanthrene were given synthetic [^{14}C]*N*-hydroxy-*p*-chloroacetanilide and the urines collected. Recovery of administered radiolabeled material in the urine was incomplete and highly variable. Recovery in the first 24 hr ranged from 27 to 63 per cent of the dose with an average of 44 ± 9 per cent. Only insignificant amounts were excreted in the second 24 hr. The incomplete recovery may be attributed in part to deacetylation with subsequent entry of the liberated [^{14}C]acetate into the general metabolic acetate pool and elimination as respiratory $^{14}CO_2$.

The urinary radioactivity could not be directly extracted into ether, indicating that nonconjugated *N*-hydroxy-*p*-

Table 1. Constant specific activity of the proposed [^{14}C]*N*-hydroxy-*p*-chloroacetanilide (metabolite 3) during sequential thin-layer chromatography*

Solvent system	R_f of *N*-hydroxy-*p*-chloroacetanilide	Specific activity of metabolite (dis./min/mg)
(1) Ether heptane (75:25)	0·40	17,890
(2) Chloroform methanol (95:5)	0·55	18,720
(3) Chloroform acetone ammonium hydroxide (15 M) (50:50:1)	0·65	18,770
(4) Chloroform methanol–ammonium hydroxide (15 M) (92:7:0·5)	0·61	17,800

* Urine from 3-methylcholanthrene-pretreated hamsters given [^{14}C]*p*-chloroacetanilide (10 mg/kg, 0·3 μCi/μmole) was digested with bacterial β-glucuronidase. Hydrolyzed metabolites (glucuronide fraction) were extracted with ether. Nonradioactive *N*-hydroxy-*p*-chloroacetanilide (250 μg) was added and the mixture subjected to thin-layer chromatography on Silica gel GF using solvent system 1 as described in the text. Metabolite 3 was isolated and its specific activity determined. Metabolite 3 was then sequentially subjected to thin-layer chromatography on Silica gel GF using the three different solvent systems shown. The specific activity of the [^{14}C]*N*-hydroxy-*p*-chloroacetanilide was determined after each thin-layer chromatographic procedure.

chloroacetanilide or *p*-chloroacetanilide was not present in the urine. However, after treatment with bacterial β-glucuronidase, over 80 per cent of the radiolabeled material was ether-extractable. Thin-layer chromatography of this ethereal extract on Silica gel GF (solvent, ether–heptane, 75:25) showed a single component which was identified as [^{14}C]*N*-hydroxy-*p*-chloroacetanilide on the basis of its chromatographic behavior. This compound had the same R_f as authentic nonradioactive *N*-hydroxy-*p*-chloroacetanilide in the three additional solvent systems listed in Table 1. In addition, the compound developed the wine-red color characteristic of hydroxamic acid-ferric chelates when sprayed with ferric chloride (0·2% in methanol) [15].

To determine if *N*-hydroxy-*p*-chloroacetanilide is a metabolite *in vivo* of *p*-chloroacetanilide, four hamsters pretreated with 3-methylcholanthrene were given ^{14}C-carbonyl-labeled *p*-chloroacetanilide and the urine was collected for 24 and 48 hr. After 24 hr, 54 ± 8 per cent of the radioactivity appeared in the urine and after 48 hr, 61 ± 7 per cent. Incomplete recovery of administered radioactivity is again attributed in part to deacetylation followed by incorporation of acetate into the general metabolite pool.

The radioactivity in urine could not be directly extracted into ether. However, after treatment with bacterial β-glucuronidase, 25 ± 2 per cent of the urinary radioactivity became ether-extractable. Since this fraction was expected to contain the majority of the *N*-hydroxy-*p*-chloroacetanilide metabolite, it was examined by thin-layer chromatography on Silica gel GF (solvent, ether–heptane, 75:25). The separation of the radiolabeled metabolites is shown in Fig. 1.

m-Hydroxy-*p*-chloroacetanilide and *o*-hydroxy-*p*-chloroacetanilide are known metabolites of *p*-chloroacetanilide [16]. Metabolite 1 and metabolite 2 (Fig. 1) were identified as *m*- and *o*-hydroxy-*p*-chloroacetanilide, respectively, on the basis of their chromatographic behavior, and their conversion to blue substances when sprayed with Gibb's reagent [17]. The *m*- and *o*-hydroxy-*p*-chloroacetanilide accounted for 38 ± 2 and 46 ± 2 per cent of the metabolites in this fraction respectively.

The radioactivity associated with metabolite 3 had the same R_f as added nonradioactive *N*-hydroxy-*p*-chloroacetanilide and contained 16 ± 1 per cent of the radioactivity found in that fraction. To verify the identity of metabolite 3 as *N*-hydroxy-*p*-chloroacetanilide, a modified isotope dilution experiment was performed (Table 1). Metabolite 3 was eluted from Silica gel and the specific activity of the ^{14}C-radiolabeled material was determined as described above. The compound was then sequentially rechromatographed using three additional solvent systems and its specific activity determined after each chromatographic step. The specific activity of the ^{14}C-radiolabel remained constant throughout the chromatographic procedures. These data confirm the identity of metabolite 3 as *N*-hydroxy-*p*-chloroacetanilide.

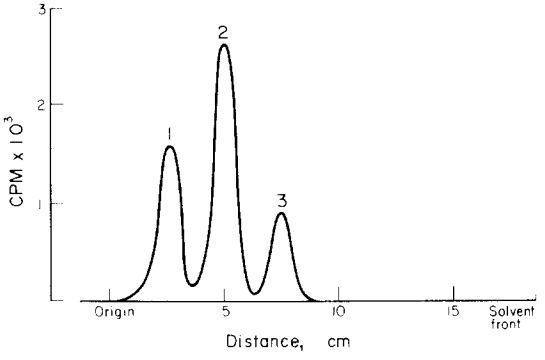

Fig. 1. Separation of metabolites of [^{14}C]*p*-chloroacetanilide by Silica gel GF thin-layer chromatography. Hamsters received [^{14}C]*p*-chloroacetanilide (10 mg/kg, 0·3 μCi, μmole, i.p.) and their urine was collected for 48 hr. Metabolites were extracted with ether after β-glucuronidase digestion. Nonradioactive *N*-hydroxy-*p*-chloroacetanilide (250 μg) was added as carrier and the mixture applied to the Silica gel GF thin-layer plate. The metabolites were fractionated using ether heptane (75:25) and identified as described in the text: 1 = *m*-hydroxy-*p*-chloroacetanilide; 2 = *o*-hydroxy-*p*-chloroacetanilide; and 3 = *N*-hydroxy-*p*-chloroacetanilide.

These findings demonstrate that *p*-chloroacetanilide is metabolized by *N*-hydroxylation in 3-methylcholanthrene-pretreated hamsters. The *N*-hydroxy-*p*-chloroacetanilide appears in the urine as a conjugate which can be hydrolyzed with bacterial β-glucuronidase. Calculation of recovery indicates that *N*-hydroxy-*p*-chloroacetanilide accounts for about 2–3 per cent of the administered dose of *p*-chloroacetanilide. In recovery experiments in which [^{14}C]*N*-hydroxy-*p*-chloroacetanilide was administered to hamsters, only 38 per cent of the dose was excreted as *N*-hydroxy-*p*-chloroacetanilide. Thus, *N*-hydroxylation of *p*-chloroace-

tanilide may account for an even greater percentage of the total metabolism.

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Metabolism of the carcinogenic bifunctional olefin oxide, 4-vinyl-1-cyclohexene dioxide, by hepatic microsomes

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Alkene oxides are known to produce malignant tumors; the bifunctional epoxides such as 4-vinyl-1-cyclohexene dioxide (1) and 1,3-butadiene dioxide have been reported to show notable carcinogenic activity on mouse skin [1]. These findings are of importance in relation to a strongly suggested role of *K*-region epoxides as metabolically formed proximate carcinogens in carcinogenicity exerted by polycyclic aromatic hydrocarbons, as they have been shown to produce malignant transformations of cells in culture [2–5]. The suggested carcinogenic mechanism involving the *K*-region epoxides, however, is an open question since previous work indicates that they have no carcinogenic activity *in vivo* [1].

Monoepoxides, including arene oxides, are detoxified by the catalytic action of either microsomal epoxide hydrolase [6, 7] (epoxide hydrase [8, 9]) or soluble epoxide-*S*-glutathione transferase [10], both of which are present in mammalian liver, to polar glycols or glutathione conjugates. However nothing is known of microsomal hydrolysis of the bifunctional epoxides, but only that no glutathione conjugation occurs with (1) *in vitro* [10] although monoepoxides such as cyclohexene oxide and styrene oxide, whose oxirane moieties are considered as its partial structures, are readily converted to the corresponding conjugates under the same conditions. This encouraged us to investigate the enzymatic hydrolysis of the carcinogen (1) by microsomal epoxide hydrolase not only from the view point of its detoxication mechanism, but also confirming whether enzymatic hydrolysis of the diepoxide follows evidence obtained by Kaubisch *et al.* [11] for structure–reactivity relationships in monoepoxides.

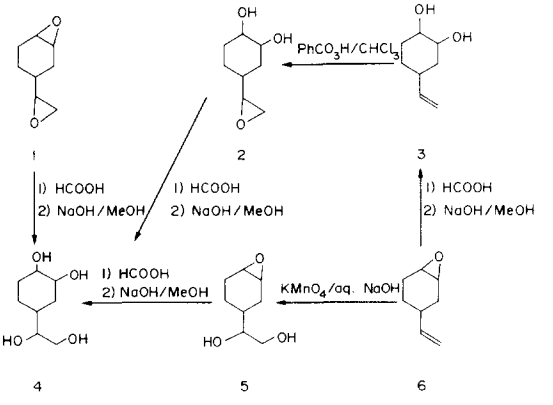

Fig. 1. Synthesis of 4-vinyl-1-cyclohexene dioxide metabolites.

The diepoxide (1) (2 mM) dissolved in acetone (0·2% v/v) was incubated at 37° with rabbit liver microsomes (1·2 mg protein/ml) in 0·1 M phosphate buffer, pH 7·4, and the