N-Hydroxyacetaminophen: A Postulated Toxic Metabolite of Acetaminophen

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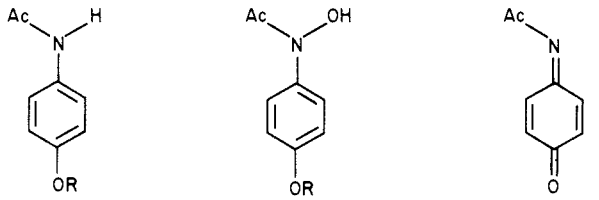
The decomposition of *N*-hydroxyacetaminophen has been shown to occur via an initial first-order dehydration step to *N*-acetyl-*p*-benzoquinone imine with a rate constant at pH 7.6 of 8.66×10^{-3} min^{-1} and a half-life of 80 min. This is followed by a complex reaction between the quinone imine and the *N*-hydroxy compound to ultimately yield *p*-nitrosophenol and acetaminophen. The glucuronide and sulfate conjugates of *N*-hydroxyacetaminophen have been observed as urinary metabolites of *N*-hydroxyacetaminophen. No N-hydroxylated metabolites were found among the metabolites of acetaminophen. These results have been interpreted to show that *N*-hydroxyacetaminophen is not a metabolite of acetaminophen. It is proposed that the hepatotoxicity and nephrotoxicity of acetaminophen are mediated by a direct oxidation of acetaminophen to the toxic reactive intermediate *N*-acetyl-*p*-benzoquinone imine by the cytochrome P_{450} dependent mixed-function oxidase system.

The mechanism of the metabolic activation of acetaminophen (1) by the cytochrome P_{450} dependent mixed-function oxidase system resulting in damage to liver and kidney continues to be the subject of controversy.[1,2] It is generally agreed that *N*-acetyl-*p*-benzoquinone imine (7) is the most likely ultimate reactive intermediate.[3] Recently, Gemborys et al.[2] have suggested that their findings are consistent with the proposal that the toxicity of acetaminophen may be mediated by its metabolic conversion to *N*-hydroxyacetaminophen, whereas, from microsomal studies, Gillette et al.[1] and Nelson et al.[4] have suggested that *N*-hydroxyacetaminophen is not an intermediate involved in the metabolism of acetaminophen.



1, R = H
2, R = glucuronide
3, R = SO_3H

4, R = H
5, R = glucuronide
6, R = SO_3H

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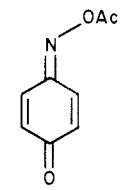

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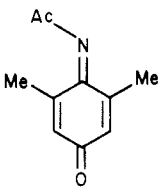

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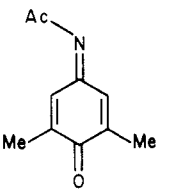

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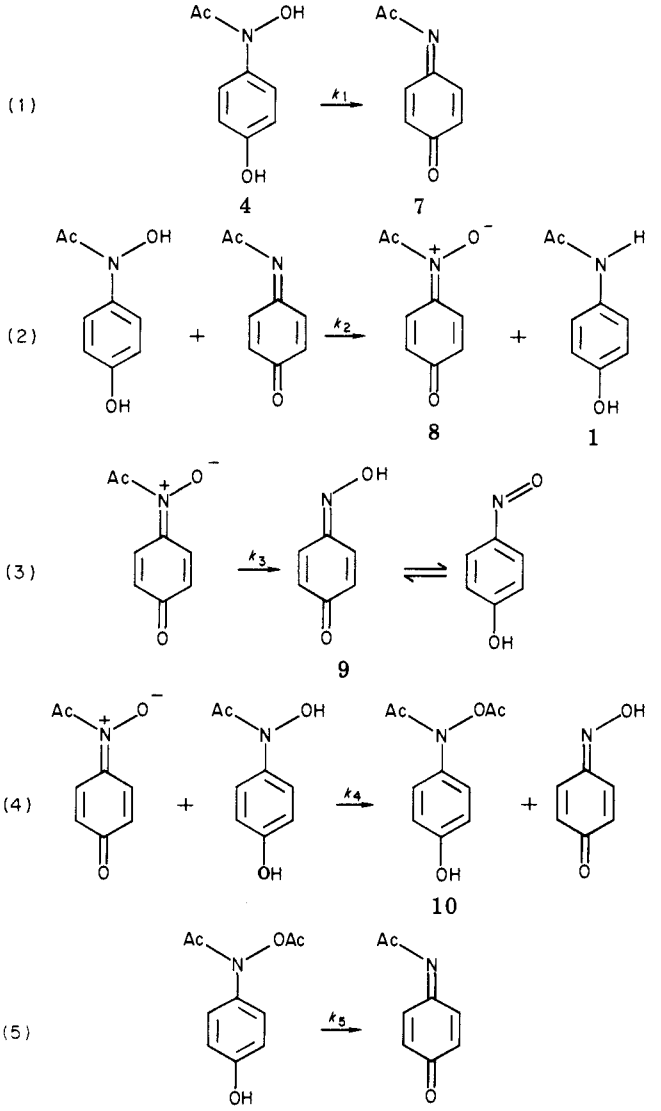
The properties[5-7] of *N*-hydroxyacetaminophen, together with the recent discussion of the mechanism of its decomposition,[2] suggest that under physiological conditions the rate of decomposition is slow enough to allow its detection as a urinary metabolite if it is formed in the metabolism of acetaminophen. Thus, no *N*-hydroxyacetaminophen was detected from microsomal incubation with acetaminophen under conditions where *N*-hydroxyacetaminophen was able to be recovered.[4] This suggestion is further supported by the detection of *N*-hydroxyacetaminophen when *N*-hydroxyphenacetin is incubated with hamster microsomes.[1] We have now reconsidered in detail the mechanism of decomposition of *N*-hydroxyacet-

Scheme I. Proposed Reaction Scheme for the Decomposition of *N*-Hydroxyacetaminophen (4)



(1) J. A. Hinson, L. R. Pohl, and J. F. Gillette, *Life Sci.*, **24**, 2133 (1979).
(2) M. W. Gemborys, G. H. Mudge, and G. W. Gribble, *J. Med. Chem.*, **23**, 304 (1980).
(3) D. J. Miner and P. T. Kissinger, *Biochem. Pharmacol.*, **28**, 3285 (1979).
(4) S. D. Nelson, A. J. Forte, and D. C. Dahlin, *Biochem. Pharmacol.*, **29**, 1617 (1980).
(5) K. Healey, I. C. Calder, A. C. Young, C. A. Crowe, C. C. Funder, K. N. Ham, and J. D. Tange, *Xenobiotica*, **8**, 403 (1978).
(6) K. Healey and I. C. Calder, *Aust. J. Chem.*, **32**, 1307 (1979).
(7) M. W. Gemborys, G. W. Gribble, and G. H. Mudge, *J. Med. Chem.*, **21**, 649 (1978).

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aminophen together with its metabolism in rats and mice.

Kinetic Studies. The decomposition of *N*-hydroxyacetaminophen has been reported to apparently follow first-order kinetics over the initial stages of the reaction[2,6] whereas the half-life for the decomposition varies with concentration.[2] An examination of the possible reactions involved suggests that the reactions shown in Scheme I (steps 1, 2, and 3) would be the simplest scheme to describe the overall decomposition, although the mechanism of the reaction is not specified.

Irreversible dehydration of *N*-hydroxyacetaminophen is proposed for the first step, since it is unlikely that a weak nucleophile such as H_2O will attack at the nitrogen. This is supported by the observation that in a detailed study of nucleophilic reactions of *N*-acetyl-2,6-dimethyl-*p*-benzoquinone imine (**12**) and *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine (**13**) the attack occurred either at the carbon of the imine function or in the ring but never on the nitrogen.[8] The second step is a second-order reaction followed by hydrolysis of the nitrone (step 3), making these steps overall irreversible.

In studying complex reactions, it is important that no further reaction can take place between the time of sampling and of analysis. While addition of aliquots of the reaction mixture to acid[2] will stop the dehydration (step 1), this change in pH may not stop the second-order reaction of *N*-hydroxyacetaminophen (**4**) with *N*-acetyl-*p*-benzoquinone imine (**7**) shown in step 2. To avoid these problems we used a high-performance liquid chromatographic method for the analysis. The time of an observation was taken on injection of the reaction mixture into the instrument.

To separate the two competing steps of the decomposition so that the rate for the dehydration step 1 could be determined, the reaction was studied under conditions in which the *N*-acetyl-*p*-benzoquinone imine (**7**) reacts with another reagent much faster than with *N*-hydroxyacetaminophen. This was achieved by carrying out the reaction in the presence of a large excess of ascorbic acid, which has been shown to quantitatively convert *N*-acetyl-*p*-benzoquinone imine to acetaminophen.[9] The rate of the dehydration of 2.0 mM *N*-hydroxyacetaminophen, as measured by the formation of acetaminophen, was independent of the concentration of ascorbic acid above 5.0 mM (Figure 1). For both 0.2 and 2.0 mM *N*-hydroxyacetaminophen, the first-order rate constant for the dehydration at pH 7.6 was 8.66×10^{-3} min^{-1}, which corresponds to a half-life of 80 min. As was observed by Gemborys et al.,[2] this reaction yielded acetaminophen quantitatively and no *p*-nitrosophenol (**9**), demonstrating that no *N*-acetyl-*p*-benzoquinone imine reacts with *N*-hydroxyacetaminophen. The dehydration was pH dependent and showed first-order kinetics. Thus, for a 2.0 mM solution at pH 7.4 the rate was 5.50×10^{-3} min^{-1} ($t_{1/2}$ = 126 min) and at pH 8.0 the rate was 2.04×10^{-2} min^{-1} ($t_{1/2}$ = 34 min).

Decomposition of *N*-hydroxyacetaminophen (**4**) was studied at pH 7.6 at 37 °C for a variety of concentrations of **4**. The reaction was followed by the rate of appearance of acetaminophen (**1**) and *p*-nitrosophenol (**9**). Injection of the reaction mixture directly into the HPLC showed the presence in low concentration of a transient intermediate of similar retention to acetaminophen, which was shown to be *p*-benzoquinone by comparison with an authentic

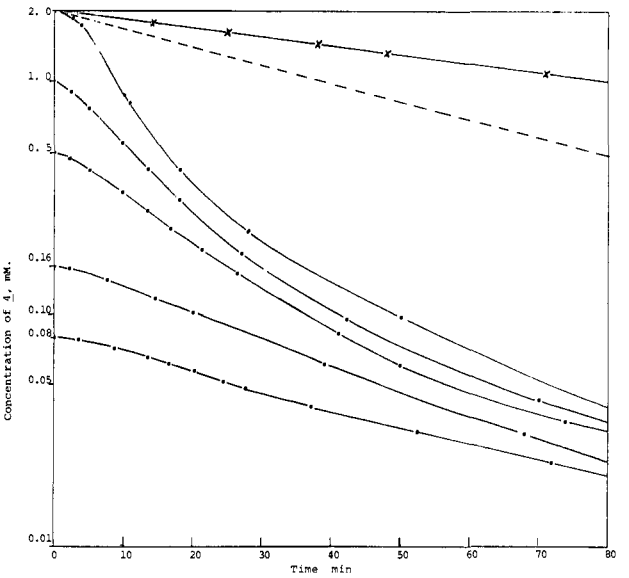


Figure 1. Semilogarithmic plot of the decompositions of various initial concentrations of *N*-hydroxyacetaminophen (**4**) (●) in the presence of excess ascorbic acid (×), calculated for twice the rate of dehydration (---).

sample. *p*-Benzoquinone was also observed in synthetic samples of *N*-acetyl-*p*-benzoquinone imine prepared by either lead tetraacetate[10] or electrochemical[3] oxidation of acetaminophen.

N-Acetoxy-*p*-benzoquinone imine (**11**) was prepared[11] and the hydrolysis was studied at pH 7.6 at 37 °C in the same buffer as was used to study the decomposition of **4**. Under these conditions, the hydrolysis of *N*-acetoxy-*p*-benzoquinone imine (**11**) to *p*-nitrosophenol (**9**) showed a pseudo-first-order rate constant of 0.15 min^{-1} ($t_{1/2}$ = 4.6 min). Thus, while no acetoxy compound (**11**) was detected, it is sufficiently stable to be detected in the decomposition of *N*-hydroxyacetaminophen (**4**). Rearrangement of the nitrone (**8**) to *N*-acetoxy-*p*-benzoquinone imine, followed by hydrolysis to *p*-nitrosophenol (**9**), probably does not occur in aqueous solution, rather the nitrone (**8**) must hydrolyze directly to *p*-nitrosophenol (**9**) (step 3). In nonaqueous solutions, however, the nitrone (**8**) will rearrange to the *N*-acetoxy compound (**11**), which can then be isolated.[6]

While the rate of decomposition of *N*-hydroxyacetaminophen (**4**) appears initially to be first order,[2,6] a closer examination of the data shows that the full course of the reaction does not follow simple first- or second-order kinetics. The decomposition of **4** at pH 7.6 for a range of initial concentrations as a semilogarithmic plot of the concentration of **4** against time (Figure 1), a reciprocal plot, or alternative plots of percentage reaction[12] reveals a complex situation.

The decomposition of *N*-hydroxyacetaminophen can be considered as competitive consecutive reactions.[12] For the simple reaction scheme involving steps 1–3 only, steps 1 and 2 can be considered thus:

$$A \xrightarrow{k_1} B$$

$$A + B \xrightarrow{k_2} C + D$$

(8) C. R Fernando, I. C. Calder, and K. N. Ham, *J. Med. Chem.*, **23**, 1153 (1980).

(9) I. A. Blair, A. R. Boobis, D. S. Davies, and T. M. Cresp, *Tetrahedron Lett.*, 21, 4947 (1980).

(10) I. C. Calder, M. J. Creek, P. J. Williams, C. C. Funder, C. R. Green, K. N. Ham, and J. D. Tange, *J. Med. Chem.*, **16**, 499 (1973).

(11) R. K. Norris and S. Sternhell, *Aust. J. Chem.*, **24**, 1449 (1971).

(12) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism", 2nd Ed., Wiley, New York and London, 1961.

For this reaction scheme, the following rates shown in eq 1–3 will apply:

$$\frac{d[A]}{dt} = -k_1[A] - k_2[A][B] \quad (1)$$

$$\frac{d[B]}{dt} = k_1[A] - k_2[A][B] \quad (2)$$

$$\frac{d[C]}{dt} = k_1[A][B] \quad (3)$$

Now for a steady-state approximation where $k_2 \gg k_1$

$$\frac{d[B]}{dt} = k_1[A] - k_2[A][B] = 0$$

Therefore, $k_1[A] = k_2[A][B]$; then, $[B] = k_1/k_2$. Thus, substituting in eq 1

$$\frac{d[A]}{dt} = -k_1[A] - k_2[A]k_1/k_2$$

Then

$$\frac{d[A]}{dt} = -2k_1[A]$$

From this it can be concluded that if $k_2 \gg k_1$, then first-order kinetics will be observed; for a simple stoichiometric second-order reaction (step 2), the maximum rate ($2k_1$) for the simple second-order reaction is shown in Figure 1. Since the rate of decomposition is significantly greater than expected, a simple second-order reaction cannot be involved. The enhanced rate at higher concentrations must be due to some type of second-order, autocatalytic effect. Reactions were carried out both under nitrogen to exclude oxygen and in the presence of the nonreducing 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy radical, but no change in the rate of decomposition was observed. This suggests a radical is not involved in the autocatalytic step.

We propose that the overall decomposition is best described by the reactions shown in Scheme I, steps 1–5. The acetylation of *N*-hydroxyacetaminophen (**4**) by the nitrone (**8**) in step 4 will form *N*-acetoxyacetaminophen (**10**), which will very rapidly lose acetic acid to form *N*-acetyl-*p*-benzoquinone imine (**7**) (step 5). The acetylation of **4** by the nitrone (**8**) would be very rapid, since hydroxamic acids are known to be excellent nucleophiles[13] and the nitrone would be expected to be a very good acetylating agent. Steps 4, 5, and 2 will then provide an autocatalytic cycle which is independent of the rate of dehydration of **4** (step 1). Further, since acetate is a much better leaving group than hydroxyl, step 5 will be much faster than step 1 and a considerable rate enhancement of the decomposition will be observed. The rate of the decomposition will be determined by either step 2 or 4, both of which are second order and involve **4**. Thus, the rate of decomposition of *N*-hydroxyacetaminophen (**4**) will show complex kinetics which will be dependent on the concentration and the relative rates of steps 1–5.

Chemistry. *N*-Hydroxyacetaminophen 4′-glucuronide [**5**; [4-(*N*-hydroxyacetamido)phenyl-β-D-glucopyranosid]-uronic acid] was prepared according to the reactions shown in Scheme II.

Methyl (4′-nitrophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate (**14**) was prepared from coupling 4-nitrophenol and methyl (tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate by a procedure similar to that of Kato et al.[14] The nitro compound **14** was reduced by aluminium amalgam in moist ether/ethyl acetate solution to give a mixture of the hydroxylamine **15** and the amine **16**, which was immediately treated with 1 equiv of ketene. This gave a mixture of the amide **18** and the desired hydroxamic acid **17**. The hydroxamic acid could not be isolated by base extraction due to the facile hydrolysis of the ester protecting groups or by formation of the copper complex due to its high solubility in all organic solvents used. However, it proved possible to separate the required hydroxamic acid (**17**) by utilizing its preferential solubility in aqueous solutions of copper acetate (4%). The protected hydroxamic acid (**17**) showed an intense absorption at 1760 cm^{-1} and a broad absorption at 1645 cm^{-1}. The ^1H NMR spectrum showed the expected AA′XX′ pattern for the aromatic protons and the sugar protons at higher field. Although no parent ion was observed in the electron-impact mass spectrum (EIMS), a strong ion for the characteristic loss of oxygen was observed at *m/e* 467. The chemical-ionization mass spectrum (CIMS), however, confirmed the presence of the hydroxamic acid, showing a parent ion at *m/e* 483.

Treatment of the hydroxamic acid (**17**) in methanol with barium hydroxymethoxide resulted in hydrolysis of the protecting groups to yield the barium salt of *N*-hydroxyacetaminophen glucuronide (**5**). The IR spectrum showed only a carbonyl at 1600 cm^{-1} due to both the carboxylic acid and the hydroxamic acid functions. This barium salt was treated with 1 equiv of H_2SO_4 to precipitate the barium, and the resulting aqueous solution was used for subsequent experiments. The structure of the *N*-hydroxyacetminophen glucuronide (**5**) was confirmed by hydrolysis with β-glucuronidase to form *N*-hydroxyacetaminophen (**4**) in high yield.

Biology. *N*-Hydroxyacetaminophen (**4**) was administered to rats and mice and acetaminophen (**1**) to mice. The mice were previously housed with pine wood-chip bedding. Animals were then housed in metabolic cages with standard feed and water ad libitum for 48 h. Urine was collected for 48 h, and at 48 h liver and kidneys were taken for histological assessment of specific toxicity, which was expressed in liver as vacuolation (1+) or centrilobular necrosis (2+, 3+, 4+) and in kidney[15] as deep cortical tubular necrosis. Tissues were graded 0 (normal) to 4+ (severe) lesions.

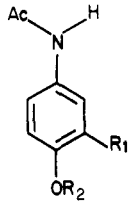
The urine was analyzed by HPLC using an ion-pair system developed for the analysis of paracetamol metabolites.[16] Authentic samples of acetaminophen (**1**), acetaminophen glucuronide (**2**), acetaminophen sulfate (**3**), acetaminophenmercapturic acid (**19**), acetaminophen cysteine conjugate (**20**), 3-(thiomethyl)acetaminophen (**21**), 3-methoxyacetaminophen (**24**), and *N*-hydroxyacetaminophen glucuronide (**5**) were available for comparison; the retention times of 3-methoxyacetaminophen glucuronide (**25**), 3-(thiomethyl)acetaminophen glucuronide (**22**), and 3-(thiomethyl)acetaminophen sulfate (**23**) were established in the course of developing the method of analysis.[16] The assignment of the peak corresponding to *N*-hydroxyacetaminophen sulfate (**6**) is tentative and was made on the basis of its chromatographic properties. Thus, typical broadening of the peak due to the hydroxamic acid function occurred together with changes in retention

(13) L. Bauer and O. Exner, *Angew Chem., Int. Ed. Engl.*, 13, 376 (1974).

(14) K. Kato, K. Yoshida, H. Tsukamoto, M. Nobunago, T. Masuya, and T. Sawada, *Chem. Pharm. Bull.*, 8, 239 (1960).

(15) I. C. Calder, C. C. Funder, C. R. Green, K. N. Ham, and J. D. Tange, *Br. Med. J.*, 4, 518 (1971).

(16) S. J. Hart, R. Tontodonati, and I. C. Calder, *J. Chromatogr.* accepted for publication.



19, R_1 = $SCH_2CH(CO_2H)NHAc$; R_2 = H
20, R_1 = $SCH_2CH(CO_2H)NH_2$; R_2 = H
21, R_1 = SMe; R_2 = H
22, R_1 = SMe; R_2 = glucuronide
23, R_1 = SMe; R_2 = SO_3H
24, R_1 = OMe; R_2 = H
25, R_1 = OMe; R_2 = glucuronide

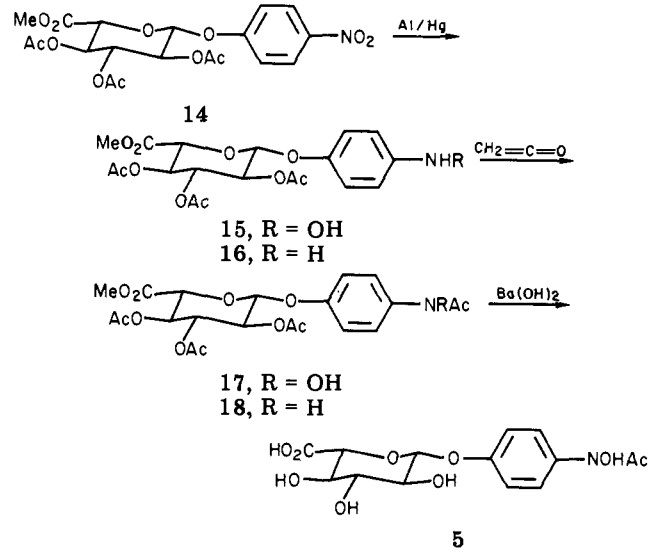
typical of a sulfate conjugate. *N*-Hydroxyacetaminophen (**4**) in aqueous solution was administered intraperitoneally to young adult male Swiss mice in a dose of 2.0 mmol/kg and to young adult male Sprague-Dawley rats in doses of 1.0 and 2.0 mmol/kg. Toxicity and urine analysis data are presented in Table I. There was no hepatotoxicity demonstrable in rats, although there was minor damage to renal tubules at a 2.0 mmol/kg dose. The mice showed low level damage to liver and no detectable change in kidney. The toxicity is comparable to that observed in previous experiments.[5] Both rats and mice excreted the glucuronide (**5**) and sulfate (**6**) conjugates of *N*-hydroxyacetaminophen in the urine. A total of between 5 and 10% of the urinary metabolites contained an *N*-hydroxy group. The remainder of the metabolites were the same as are normally observed from acetaminophen, although the proportions were significantly different.

Female Swiss mice are particularly susceptible to acetaminophen-induced hepatic necrosis. Thus, the urinary metabolites and toxicity were examined closely in young adult female Swiss mice at doses of acetaminophen of 2.0 mmol/kg intravenously and 10 mmol/kg intragastrically (Table I). At a dose of 2.0 mmol/kg no toxicity was observed, while at 10 mmol/kg, grade 3 hepatic necrosis was observed with no change in the kidney. The results of the analysis of the urinary metabolites are shown in Table I. No *N*-hydroxyacetaminophen glucuronide (**5**) or *N*-hydroxyacetaminophen sulfate (**6**) was observed to the level of sensitivity of 0.5 μmol/mL in urine. Further, no *N*-hydroxy metabolites of acetaminophen have been detected by this method as urinary metabolites from acetaminophen (**1**) in any strain of rat or mouse with any route of administration studied in our laboratory.

Discussion

These results demonstrate that the decomposition of *N*-hydroxyacetaminophen (**4**) is composed of an initial first-order dehydration step, followed by a second-order reaction of *N*-acetyl-*p*-benzoquinone imine (**7**) with **4**. An autocatalytic step involving acetylation of *N*-hydroxyacetaminophen (**4**) by the nitrone (**8**) increases the rate of the decomposition at higher concentrations. The complex reaction scheme for the decomposition involves steps 1–5 (Scheme I). On the basis of this scheme the stability of *N*-hydroxyacetaminophen (**4**) will be primarily dependent on step 1. Thus, **4** will be relatively stable from pH 1 to 7 where it exists substantially in the un-ionized form; at high pH it is also stable presumably because both ionizable groups are dissociated and at intermediate pH 7 to 12 it is unstable and rapidly decomposes. In the presence of reducing agents or nucleophiles the concentration of *N*-acetyl-*p*-benzoquinone imine will be lowered, thus reducing the contribution of the autocatalytic steps to the decomposition. This will change the ratio of products and increase the half-life observed for the decomposition, accounting for the effects of additives observed by Gemborys et al.

Scheme II. Synthesis of *N*-Hydroxyacetaminophen Glucuronide (**5**)



The dehydration of *N*-hydroxyacetaminophen (**4**) is a first-order reaction with a rate constant of 8.66×10^{-3} min^{-1} at pH 7.6, giving a half-life of 80 min for this step. This dehydration reaction alone will be observed where a reducing agent or a nucleophile reacts rapidly with *N*-acetyl-*p*-benzoquinone imine (**7**), and under these conditions *N*-hydroxyacetaminophen (**4**) will be relatively stable. These conditions would prevail in the cell if *N*-hydroxyacetaminophen were formed from acetaminophen, and the half-life should be 80 min or greater, depending on the pH. In fact, when formed in the cell, *N*-hydroxyacetaminophen (**4**) would be at such a low concentration that any contribution from second-order reactions, such as step 4, would be negligible. The actual lifetime observed would be dependent on the rate of conjugation. Conjugation of the hydroxamic acid hydroxyl would rapidly lead to *N*-acetyl-*p*-benzoquinone imine (**7**), whereas reaction of the 4′-hydroxyl will form the glucuronide (**5**) or sulfate (**6**) conjugates.

If *N*-hydroxyacetaminophen (**4**) is a metabolite of acetaminophen (**1**), although in lower concentration, all the urinary metabolites of **4** would be expected to be present in the urine of animals administered acetaminophen (**1**).

After administration of *N*-hydroxyacetaminophen (**4**) to rats and mice, both the glucuronide and sulfate conjugates were observed in a total yield of 5–10%. This observation established that *N*-hydroxyacetaminophen (**4**) is sufficiently stable in the biological situation for it to be able to enter a cell and to undergo conjugation reactions. As well there were large amounts of the acetaminophen-mercapturic acid and cysteine conjugates formed, as expected from the reaction of glutathione with *N*-acetyl-*p*-benzoquinone imine (**7**). Acetaminophen (**1**) may be formed via direct reduction of *N*-hydroxyacetaminophen (**4**) as has been established for *N*-hydroxyfluorenylacetamide[17] or alternatively via reduction of *N*-acetyl-*p*-benzoquinone imine (**7**) with biological reducing agents. Such reduction will then result in the excretion of acetaminophen and its glucuronide and sulfate conjugates.

Since no *N*-hydroxyacetaminophen glucuronide (**5**) or sulfate (**6**) can be detected in the urine of mice after ad-

(17) L. A. Wheeler, F. B. Soderberg, and P. Goldman, *Cancer Res.*, 25, 2962 (1975).

Table I. Metabolic Disposition and Toxicity of *N*-Hydroxyacetaminophen (4) and Acetaminophen (1)

compd	4	4	4	1	1
dose, mmol/kg	1.0	2.0	2.0	2.0	10.0
route of adm	ip[a]	ip	ip	iv[b]	ig[c]
species	rat	rat	mouse	mouse	mouse
sex	male	male	male	female	female
no. of animals	2	4	4	4	2
toxicity in liver	0	0	2+	2+	3+
toxicity in kidney	0	+	0	0	0
	% Dose Excreted in 0–48 h[e]				
	70.9 ± 5.1	55.0 ± 13.6	41.5[d]	59.8 ± 6.8	58.8 ± 8.7
Metabolic Disposition:	% of Total Excreted Metabolites[e]				
acetaminophen glucuronide (2)	10.8 ± 3.8	18.0 ± 1.4	25.9	64.0 ± 1.3	58.4 ± 2.4
acetamionphen sulfate (3)	51.3 ± 11.9	47.1 ± 5.2	5.3	13.8 ± 1.1	13.3 ± 0.6
acetaminophen (1)	7.0 ± 3.8	5.0 ± 2.6	4.5	11.9 ± 2.2	12.1 ± 2.9
acetaminophenmercapturic acid (19)	14.5 ± 1.5	13.2 ± 3.9	6.1	3.5 ± 1.6	3.8 ± 1.6
acetaminophen cysteine conjugate (20)	0	1.9 ± 1.9	32.0	2.9 ± 3.4	10.4 ± 2.1
3-(thiomethyl)acetaminophen sulfate (23)	5.1 ± 3.2	9.0 ± 5.3	15.9	3.3 ± 1.0	1.5 ± 0.7
3-(thiomethyl)acetaminophen glucuronide (22)	1.9 ± 1.0	2.0 ± 1.1	4.1	0.7 ± 0.4	0.6 ± 0.3
3-methoxyacetaminophen glucuronide (25)	0	0	0	0.0	0.0
N-hydroxyacetaminophen glucuronide (5)	1.5 ± 1.1	2.3 ± 2.1	3.4	0.0	0.0
N-hydroxyacetaminophen sulfate (6)	8.1 ± 3.0	2.7 ± 1.8	3.0	0.0	0.0

[a] Intraperitoneal. [b] Intravenous. [c] Intragastric. [d] Five animals in one cage. Composite urine sample. [e] Plus or minus SEM.

ministration of acetaminophen, it can be concluded that no N-hydroxylation of acetaminophen occurs. The most likely metabolic scheme to account for the observed hepatotoxicity and nephrotoxicity of acetaminophen is one in which acetaminophen is oxidized directly to *N*-acetyl-*p*-benzoquinone imine (7) by the cytochrome P_{450} dependent mixed function oxidase system.

Consideration of the decomposition of *N*-hydroxyacetaminophen (4) shows that *N*-acetyl-*p*-benzoquinone imine (7) has properties consistent with its proposed role in the metabolic scheme. Firstly, *N*-acetyl-*p*-benzoquinone imine (7) is sufficiently stable in aqueous medium to be formed at the site of metabolism and then to migrate to a site where it can react with either glutathione or cell protein; the latter may result in cell damage. This can be concluded from the fact that the *N*-acetyl-*p*-benzoquinone imine (7) does not hydrolyze immediately to *p*-benzoquinone when formed in water but rather reacts preferentially with *N*-hydroxyacetaminophen (4) even at low concentrations of 7. Secondly, since *N*-acetyl-*p*-benzoquinone imine (7) does not accumulate in the reaction mixture but rather reacts with any available substrate, it can be considered as a reactive intermediate. In all cases studied, reactions of *N*-acetyl-*p*-benzoquinone imine with ascorbic acid or thiol is preferred to reaction with the initial *N*-hydroxy compound. Thus, it would not be expected that *N*-acetyl-*p*-benzoquinone imine (7) could be detected in metabolic studies involving microsomes with their associated thiol groups and reducing species such as NADPH. Thirdly, the reactions shown in Scheme I and the kinetics observed account for all the results reported to date, and there is no need to postulate radical species to account for any step of the decomposition.

The oxidation of organic molecules by the cytochrome P_{450} mixed-function oxidase system involves an active oxygen species.[18] The reaction may proceed via an insertion process similar to that observed for carbenes, resulting in the direct formation of hydroxyl groups or arene oxides. Alternatively, an initial hydrogen abstraction may occur, followed by subsequent reaction with an oxygen species to give the product. In the case of acetaminophen, the first mechanism would yield *N*-hydroxyacetaminophen and, therefore, seems unlikely in the light of the evidence presented in this paper. In the case of initial hydrogen abstraction from acetaminophen, it is likely that a second hydrogen will be lost before the oxygenation can occur. This would result in the formation of *N*-acetyl-*p*-benzoquinone imine directly.

(18) V. Ullrich, *Top. Curr. Chem.*, 83, 67 (1979).

Experimental Section

Melting points were determined on a Kofler hot stage microscope. TLC was carried out on Merck silica gel GF_{254}, 0.25 mm for analytical and 1 mm for preparative plates. Light petroleum refers to the fraction of bp 40–60 °C. Acetonitrile was purified by the procedure of Burfield et al.[19] Dimethylaminobenzaldehyde reagent was a 1% solution in ethanol containing 0.1 N HCl.

β-Glucuronidase/aryl sulfatase was obtained from Boehringer-Mannheim, GmbH, Hamburg.

Microanalyses were performed by the Australian Microanalytical Service, Melbourne.

The NMR spectra were recorded on a Perkin-Elmer R-12, a JEOL FX-100, or a Varian HA-100 spectrometer at 60 and 100 MHz, respectively, with tetramethylsilane as internal standard. Mass spectra were obtained on an AEI MS9 high-resolution mass spectrometer at 70 eV. Chemical-ionization mass spectra were obtained on a VG70-70 spectrometer, with isobutane as the reagent gas. The peak intensity is given as a percentage of the base peak. Infrared spectra were determined as KBr disks on a Perkin-Elmer 457 spectrometer, and ultraviolet spectra were determined on a Unicam SP-800 ultraviolet spectrometer. Optical rotations were recorded on a Perkin-Elmer 141 polarimeter as 1% w/v solutions.

***N*-Hydroxyacetaminophen** (4) was prepared as previously described[6] as white needles, mp 120–122 °C dec (lit.[6] 120–122 °C dec).

***N*-Acetoxy-*p*-benzoquinone imine** (11) was prepared by the method of Norris and Sternhell[11] as yellow crystals, mp 106–107 °C (lit.[11] 109 °C).

High-performance liquid chromatography (HPLC) was carried out with a Spectra-Physics 3500 B high-pressure liquid chromatograph fitted with a Waters U6K loop injector and a Waters dual-wavelength absorbance detector at wavelengths of 254 and 280 nm. Chromatography was done at ambient temperature, and mobile phases were filtered and degassed before use.

Method 1. For the analysis of metabolites of *N*-hydroxyacetaminophen (4), a high-resolution gradient ion-pair system on a Waters μBondapak C_{18} column (0.4 × 30 cm) was used.[16] Solvent A was composed of 0% methanol in water containing 0.005 M tetrabutylammonium hydroxide, 0.01 M Tris, and 0.005 M EDTA

(19) D. R. Burfield, K. H. Lee, and R. H. Smithers, *J. Org. Chem.*, 42, 3060 (1977).

buffered to pH 7.2 with phosphoric acid. Solvent B was identical, except that it contained 50% methanol. The separation was begun with solvent A at 2 mL/min. After the injection of filtered urine (0.5 μm) into the high-performance liquid chromatograph, the methanol concentration was increased to 50% linearly over 18 min after an initial delay of 4 min. After pure solvent B had been flowing for 10 min, the column was regenerated by using a fast reverse program and reequilibration with solvent A for about 20 min. Analysis of samples thus took approximately 1 h. Response factors were determined by correlating the peak area to known concentrations of standard solutions.

Method 2. For the analysis of decomposition samples of *N*-hydroxyacetaminophen (4), a faster HPLC separation than above was desired. This was achieved on Water's Radial Pak A reverse-phase columns using an isocratic solvent of 15% methanol in potassium phosphate buffer (0.05 M) at pH 6.0. A flow rate of 3.2 mL/min was used and the following retention times were observed: *p*-nitrosophenol (9), 2.2 min; acetaminophen (1), 3.6 min; *p*-benzoquinone, 3.9 min; *N*-hydroxyacetaminophen (4), 4.2 min; *N*-acetoxy-*p*-benzoquinone imine (11), 23.5 min.

Decomposition of *N*-Hydroxyacetaminophen (4). The standard conditions for following the reactions of 4 were incubation at 37 °C in 0.2 M potassium phosphate buffer at pH 7.6 with a slow stream of nitrogen passing through the solution. Standard stock solutions of *N*-hydroxyacetaminophen (4) in dilute phosphoric acid (0.002 M) and ascorbic acid in phosphate buffer, pH 7.6, were prepared. For kinetic studies the start of the reaction was taken when appropriate aliquots of the stock solutions were mixed. The time of an observation was taken on injection of the reaction mixture into the instrument. The decomposition was examined at least twice at each concentration studied using different sampling times in successive runs.

Analysis of Metabolites of *N*-Hydroxyacetaminophen (4) and Acetaminophen (1). *N*-Hydroxyacetaminophen (4) and acetaminophen (1) as the sodium salt were administered to animals in aqueous solution. The urine was collected in glass metabolic cages which allowed the separation of urine and feces. The urine was filtered through millipore filters (0.5 μm) and aliquots were injected directly into the high-performance liquid chromatograph.

[4-(*N*-Hydroxyacetamido)phenyl β-D-glucopyranosid]uronic Acid (5). Methyl tetra-*O*-acetyl-β-D-glucopyranuronate was prepared by the method of Bollenback et al.[20] and was recrystallized from ethanol/chloroform as white needles (23%): mp 177–178 °C (lit.[20] 176.5–178 °C); ^{1}H NMR ($CDCl_3$) δ 2.0 (s, 9 H), 2.1 (s, 3 H), 3.7 (s, 3 H), 4.2 (m, 1 H), 5.3 (m, 3 H), 5.8 (m, 1 H).

Methyl 2,3,4-tri-*O*-acetyl-1-bromo-α-D-glucopyranuronate was prepared by the method of Bollenback et al.[20] The crude product was dissolved in the minimum volume of chloroform and an excess of ethanol was added. The solution was stored at −20 °C for 24 h, during which the product crystallized as white prisms (53%): mp 107–108 °C (lit.[20] 106–107 °C); ^{1}H NMR ($CDCl_3$) δ 2.0 (s, 6 H), 2.1 (s, 3 H), 3.8 (s, 3 H), 4.6 (d, J = 12 Hz, 1 H), 4.9 (dd, J = 9 and 4 Hz, 1 H), 5.3 (J = 10 Hz, 1 H), 5.7 (t, J = 10 Hz, 1 H), 6.7 (d, J = 4 Hz, 1 H).

Methyl [4′-nitrophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]uronate (14) was prepared by the method of Kato et al.[14] using freshly distilled acetonitrile[19] and recrystallized 4-nitrophenol. Recrystallization of the crude product from chloroform/ethanol gave 14 as white needles (57%): mp 153–154 °C (lit.[14] 151–152 °C); ^{1}H NMR ($CDCl_3$) δ 2.0 (s, 9 H), 3.7 (s, 3 H), 4.2 (m, 1 H), 5.3 (m, 4 H), 7.1 (d, J = 9 Hz, 2 H), 8.2 (d, J = 9 Hz, 2 H).

Methyl [4-(*N*-Hydroxyacetamido)phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]uronate (17). 14 (1 g, 2.2 mmol) was dissolved in a mixture of ether (150 mL) and ethyl acetate (50 mL) and freshly prepared aluminium amalgam[21] (0.6 g foil) was added. The temperature of the stirred solution was maintained between 15 and 20 °C with an ice bath. The reduction was monitored by TLC (chloroform/ethanol, 9:1) every 5 min, using plates prepared on microscope slides. The mixture was filtered through a fine sintered glass funnel just before all the nitro compound (R_f 0.9) had reacted.

The reaction mixture was then treated with 1 equiv of ketene gas (2.2 mmol) as judged by a negative color test of residual amine or hydroxylamine with dimethylaminobenzaldehyde. Acetylation caused the formation of a white precipitate. The solvent was removed in vacuo, and the white solid was added to an aqueous solution of cupric acetate monohydrate (4%) and vigorously stirred for 30 min.

The insoluble amide (18) was collected by filtration through a fine sintered glass funnel, and to the filtrate was added an excess of EDTA (2.5 g) and chloroform (100 mL). The mixture was then shaken in a separating funnel until the aqueous phase was bright blue, and the chloroform layer was collected. The aqueous solution was extracted with a further 3 volumes of chloroform and all the extracts were combined, dried (Na_2SO_4), and evaporated in vacuo. The crude product was recrystallized from propan-2-ol to give 17 as white prisms (327 mg, 31%): mp 127–128 °C; $[\alpha]^{22}_D$ −27.9° (c 1, $CHCl_3$); IR ν_{max} 3440 (br), 1760 (s), 1645 (br m), 1510, 1375, 1230, 1040 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 9 H), 2.15 (s, 3 H), 3.74 (s, 3 H), 5.29 (m, 1 H), 5.32 (m, 4 H), 7.04 (d, J = 9 Hz, 2 H), 7.33 (d, J = 9 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 20.5 (CH_3CO_2), 24.3 (CH_3CON), 53.0 (CH_3OCO), 68.9, 71.0, 71.7, 72.5 (C2–C5), 98.8 (C1), 117.5 (C3′, C5′), 121.6 (C2′, C6′), 133.5 (C1′), 153.2 (C4′), 166.7, 169.2, 169.3, 170.0; mass spectrum (reagent gas isobutane; CI), m/e 483 (M, 2) 467 (M − O, 9), 317 (33), 257 (11), 168 (2), 152 (15) and 43 (100). Anal. ($C_{21}H_{25}NO_{12}$) C, H, N.

Methyl [4-(Acetamido)phenyl β-D-glucopyranosid]uronate (18). The material which was insoluble in the cupric acetate solution of the above reaction was recrystallized from propan-2-ol to give methyl [4-(acetamido)phenyl β-D-glucopyranosid]uronate (18) as white prisms (420 mg, 41%): mp 213.5–214.5 °C (lit.[22] 209–210 °C); $[\alpha]^{21}_D$ −35.2° (c 1, $CHCl_3$); IR ν_{max} 3300, 1760, 1670, 1510, 1375, 1225, 1050 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 6 H), 2.07 (s, 3 H), 2.15 (s, 3 H), 3.73 (s, 3 H), 4.19 (m, 1 H), 5.10 (m, 1 H), 5.30 (m, 3 H), 6.94 (d, J = 9 Hz, 2 H), 7.41 (d, J = 9 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 20.6 (CH_3CO_2), 24.3 (CH_3CON), 53.0 (CH_3OCO), 69.2, 71.1, 71.8, 72.5 (C2–C5), 99.6 (C1), 117.7 (C3′, C5′), 121.6 (C2′ C6′), 133.7 (C1′), 153.2 (C4′), 167.0, 168.5, 169.3, 170.1.

Barium Methoxide [4-(Barium methoxyacetylhydroxamato)phenyl β-D-glucopyranosid]uronate. To a solution of [4-(*N*-hydroxyacetamido)phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyransid]uronate (17; 100 mg, 0.2 mmol) in methanol (20 mL) was added a solution of barium hydroxymethoxide in methanol (1 mL; 71 mg of Ba/mL), and the mixture was stored at 4 °C overnight. The white barium salt was collected by filtration through a fine sintered glass funnel, washed with water and ethanol, and finally dried in a vacuum dessicator: yield 88 mg (62%). The barium salt could not be recrystallized and was soluble only in acetic acid in which it decomposed. The product decomposed at 180 °C but did not melt below 360 °C. IR ν_{max} 3420 (br), 1600 (br), 1510, 1420, 1230, 1070 cm^{-1}. Anal. ($C_{16}H_{21}NO_{11}Ba_2$) C, H, N, Ba.

[4-(*N*-Hydroxyacetamido)phenyl β-D-glucopyranosid]uronic Acid (5). The bis barium salt of 5 (0.678 mg, 1 μmol) was added to H_2SO_4 (1 mM, 2 mL) and stood at 4 °C for 2 h. The fine white precipitate of $BaSO_4$ was removed by filtration through a small column of supercel and the filtrate was transferred to a 10-cm^3 volumetric flask. The column was washed with water and the washings were transferred into the flask to a final volume of 10.0 mL. This provided a solution of 5 (0.1 mM): UV λ_{max} 233 nm (ε 4.03).

Hydrolysis of 5 with β-Glucuronidase. β-Glucuronidase/aryl sulfatase (50 μL) was added to the solution obtained above and incubated at 37 °C for 1 h. HPLC indicated quantitative hydrolysis of 5 to *N*-hydroxyacetaminophen (4).

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(20) G. N. Bollenback, J. W. Long, D. G. Benhamin, and J. A. Lindquist, *J. Am. Chem. Soc.*, 77, 3310 (1955).

(21) A. I. Vogel, "Practical Organic Chemistry", 3rd ed., Longman, New York, 1955.

(22) J. Kiss, K. Noack, and R. D'Souza, *Helv. Chim. Acta*, 58, 301 (1975).