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PRELIMINARY COMMUNICATIONS

LACK OF EVIDENCE FOR N-HYDROXYACETAMINOPHEN AS A REACTIVE METABOLITE OF ACETAMINOPHEN IN VITRO

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The widely used analgesic acetaminophen causes liver necrosis in man and experimental animals (1,2). Metabolic activation of acetaminophen was originally postulated to occur via N-oxidation to N-hydroxyacetaminophen (N,4'-dihydroxyacetanilide) which dehydrated to the arylating agent, N-acetyl-p-benzoquinone imine (3). Subsequently, N-hydroxyacetaminophen has been synthesized in crystalline form and has been found to have both chemical and toxicological characteristics consistent with its postulated role as an intermediate in the metabolism of acetaminophen to an arylating agent (4-7).

However, recent kinetic evidence has appeared (8) which suggests that the arylating metabolite of acetaminophen does not arise via the formation of N-hydroxyacetaminophen in microsomal incubations. In order to determine more directly whether or not N-hydroxyacetaminophen is formed as a metabolite of acetaminophen in microsomal incubations, we carried out trapping experiments using a carrier pool of N-hydroxyacetaminophen. The experiments were similar to those described by Jerina et al. (9) for determining the intermediacy of 1,2-naphthalene oxide as an intermediate in the microsomal hydroxylation of naphthalene.

Microsomal incubations were carried out essentially as described by Hinson et al. (8) with the exception that we used microsomes prepared from the livers of Swiss-Webster mice (20-25 g, Washington State University Laboratory of Animal Medicine, Pullman, WA). The recovery of N-hydroxyacetaminophen from control incubations ranged from 67-71% down to concentrations of 100μM. Therefore, in a typical experiment, microsomal incubations in a total volume of 3 ml contained 6 mg microsomal protein, 6 mg NADPH, 3 μmoles EDTA, 150 μmoles sodium phosphate, pH 7.4, 0.5 μmole of N-hydroxyacetaminophen and 3 μmoles of ^{3}H-acetaminophen (120 dpm/picomole).

The radiolabeled acetaminophen (New England Nuclear, Boston MS) was purified by a combination of chromatography on Analtech Silica Gel GF thin layer plates (250μ thickness) using ether as developing solvent, and by hplc using conditions previously described (10). N-Hydroxyacetaminophen was prepared by hydrogenolysis of 4-benzyloxy-N-hydroxyacetanilide as originally outlined by R.S. Andrews (Sterling-Winthrop Laboratories, United Kingdom). Our synthetic sample of N-hydroxyacetaminophen had m.p. (121-123°C with decomposition) and

spectral characteristics (proton nmr, ir and e.i.m.s.) virtually identical with those reported by others (5,7). Gas chromatography of the silyl derivative on a 1.8m x 2 mm i.d. glass column packed with 3% SE-30 showed the presence of a small amount (1-2%) of acetaminophen in the sample.

N-hydroxyacetaminophen was reisolated from the microsomal incubations by ethyl acetate extraction and analyzed by hplc as its ferric chelate (8). Acetaminophen had a retention time elution range of 3-4 min and N-hydroxyacetaminophen a retention time elution range of 5.3-6.5 min under the conditions of our assay. The N-hydroxyacetaminophen fraction was collected (5-7 min) in scintillation vials, 15 ml of ACS scintillation cocktail was added, and radioactivity was determined by scintillation spectroscopy on a Beckman LS 7500 instrument with automatic quench correction. Irreversible binding was determined as previously described (11) after initial precipitation of the protein with methanol.

In order to insure that we were collecting N-hydroxyacetaminophen, samples of the hplc purified iron chelated material (representing a total of approximately 100 µg of N-hydroxyacetaminophen) were combined with 10 ml of 1 mM EDTA solution. The uncomplexed N-hydroxyacetaminophen was then reisolated by extraction into ethyl acetate. Direct probe chemical ionization mass spectrometry of the sample after incubation, hplc purification, and reextraction gave virtually the same spectrum as authentic N-hydroxyacetaminophen (Fig. 1).

Results of the irreversible binding of radiolabel to the microsomal protein show that the presence of N-hydroxyacetaminophen has little effect on the binding reaction (Fig. 2). More importantly, no radioactivity above background levels could be found in the N-hydroxyacetaminophen reisolated from the incubations at any time point. Whereas the irreversible binding of radiolabel reached levels of 11-13 nmoles in 15 minutes compared to 0.6 nmole in the absence of NADPH, we could not detect any activity above background levels (8-17 picomoles) in the N-hydroxyacetaminophen fraction isolated by hplc. Therefore, under the conditions of these carrier trapping experiments, N-hydroxyacetaminophen could only be formed as a metabolite of acetaminophen if it did not dissociate from the enzyme-product complex. Based on the finding that N-hydroxyphenacetin can be metabolized to detectable levels of N-hydroxyacetaminophen by microsomal oxygenases (8), we feel that lack of dissociation of N-hydroxyacetaminophen from these enzymes is unlikely.

From this study we conclude that the electrophilic metabolite of acetaminophen which irreversibly binds to mouse microsomal protein is not formed from N-hydroxyacetaminophen. However, oxidation of the amide nitrogen of acetaminophen does appear to be necessary for hepatotoxicity to develop (12) and recent evidence strongly implicates N-acetyl-p-benzoquinone imine as an arylating metabolite of acetaminophen (13). Thus, other mechanisms should be considered for the formation of the arylating metabolite(s) of acetaminophen.

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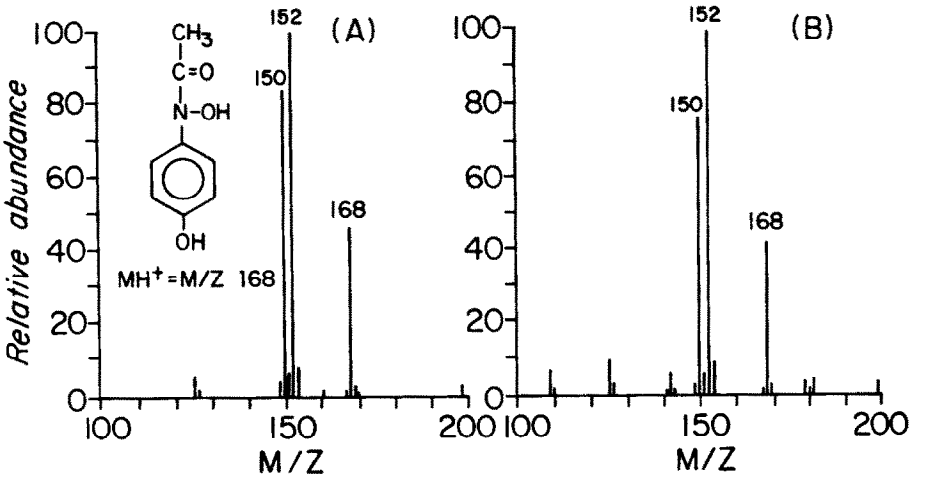

Figure 1: (A) Chemical Ionization mass spectrum of synthetic N-hydroxyacetaminophen. (B) Chemical ionization mass spectrum of synthetic N-hydroxyacetaminophen isolated by hplc from incubations of mouse liver microsomes after a 15-minute incubation period. Samples were assayed by direct probe insertion on a Biospect Chemical Ionization Quadrupole Mass Spectrometer (Scientific Research Corporation, Baltimore, MD) using methane as reagent gas with a source pressure of 0.5 torr and a source temperature of 180°C.

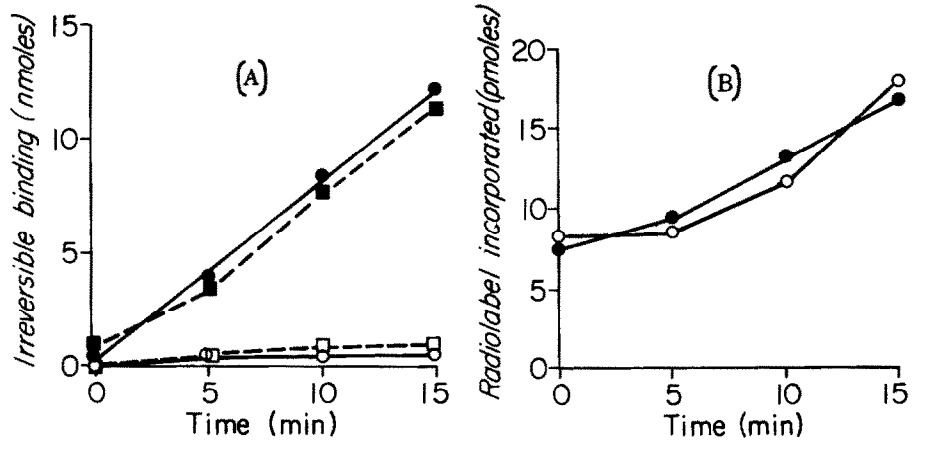

Figure 2: Time course of (A) the irreversible binding of radiolabel from ^{3}H-acetaminophen to microsomal protein and (B) the incorporation of radiolabel into a carrier pool of N-hydroxyacetaminophen. Data points represent the average values from 2 separate experiments with duplicate samples in each experiment. Values did not vary more than ± 5% for irreversible binding determinations and ± 11% for radioactive incorporation determinations.

● — Acetaminophen + N-Hydroxyacetaminophen + NADPH
○ — Acetaminopehn + N-Hydroxyacetaminophen - NADPH
■ -- Acetaminophen - N-Hydroxyacetaminophen + NADPH
□ -- Acetaminophen - N-Hydroxyacetaminophen - NADPH

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