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# 2-ACETAMIDO-*p*-BENZOQUINONE: A REACTIVE ARYLATING METABOLITE OF 3′-HYDROXYACETANILIDE

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**Abstract**—The covalent binding to protein of 3′-hydroxyacetanilide (3HAA), its primary metabolite 2′,5′-dihydroxyacetanilide (2,5DHAA), and a putative secondary metabolite thereof, 2-acetamido-*p*-benzoquinone (APBQ), was studied in hepatic microsomal preparations from phenobarbital-pretreated mice. All compounds were found to bind irreversibly to microsomal protein, APBQ being by far the most effective member of the group. In the case of 3HAA, binding was dependent upon the presence in incubation media of the co-factor NADPH, indicating that metabolism of 3HAA was necessary for the generation of a reactive intermediate. In contrast, NADPH decreased by more than 2-fold the binding of both 2,5DHAA and APBQ. The free radical spin-trapping agent α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) did not reduce the binding of 3HAA to protein. These results support the contention that metabolic activation of 3HAA is a two-step process which involves initial aromatic hydroxylation to give the substituted hydroquinone, 2,5DHAA, followed by a second oxidation reaction (which may not be enzyme-mediated) to produce the benzoquinone derivative, APBQ. This quinone is a reactive, electrophilic intermediate which may either undergo reduction back to 2,5DHAA or bind covalently to cellular macromolecules.

3′-Hydroxyacetanilide (3HAA)† is a positional isomer of acetaminophen (4′-hydroxyacetanilide) which also possesses analgesic and antipyretic properties [1, 2]. Unlike acetaminophen, however, 3HAA is not hepatotoxic to hamsters [3] or mice [4] *in vivo* even though the two isomers have been found to bind covalently to liver protein to a similar extent [5]. This striking lack of correlation between covalent binding and toxicity with 3HAA prompted us to carry out an investigation into the metabolic fate of 3HAA *in vitro* and to compare the covalent binding characteristics of this compound with those of acetaminophen [6]. The results of this study showed that 3HAA is metabolized in mouse liver microsomal preparations to 2′,5′-dihydroxyacetanilide (2,5-DHAA), 3′,4′-dihydroxyacetanilide (3,4DHAA), and 2′,3′-dihydroxyacetanilide, and that four times more 3HAA than acetaminophen becomes bound irreversibly to protein under comparable incubation conditions. Moreover, experiments using ascorbic acid, GSH and model proteins indicated that the mechanism by which 3HAA binds covalently to cellular constituents probably involves further oxidation of one or more of the primary 3HAA metabolites to an electrophilic intermediate which reacts with sulfhydryl groups. Since 3,4DHAA does not become bound to protein to an appreciable extent in microsomal incubations [7], it was concluded that a likely candidate for the reactive metabolite of 3HAA was 2-acetamido-*p*-benzoquinone (APBQ), the quinone derivative resulting from two-electron oxidation of 2,5-DHAA. The work reported in this paper provides further evidence in support of this hypothesis.

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† Abbreviations: 3HAA, 3′-hydroxyacetanilide; 2,5DHAA, 2′,5′-dihydroxyacetanilide; 3,4DHAA, 3′,4′-dihydroxyacetanilide; APBQ, 2-acetamido-*p*-benzoquinone; GSH, reduced glutathione; TMS, trimethylsilyl; BSTFA, *bis*-(trimethylsilyl)trifluoroacetamide; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; MU, methylene unit; and HPLC, high-performance liquid chromatography.

## MATERIALS AND METHODS

### *Materials*

Potassium nitrosodisulfonate was purchased from the Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were obtained from commercial sources as listed previously [6].

### *Instrumentation*

Proton NMR spectra (80 MHz) were recorded using a modified Varian CFT-20 spectrometer. Electron impact mass spectra were obtained with a VG 70-70H instrument, coupled directly to a Hewlett–Packard model 5710A gas chromatograph and operated in the GC-MS mode [6].

### *Syntheses*

*APBQ*. This compound was prepared by oxidation of 3HAA (1.0 g, 6.6 mmoles) with potassium nitrosodisulfonate (5.3 g, 19.8 mmoles) according to a published method [8]. Following recrystallization from dichloromethane/hexane (1:1), the yield of APBQ was 0.68 g (21%), m.p. 145–147° with decomposition (lit. [8] 144–147°). MS: *m/z* 165 ($M^{+\cdot}$, 100%), 149 ($[M—O]^{+\cdot}$, 7%), 137 ($[M—CO]^{+\cdot}$, 6%), 123 ($[M—CH_2CO]^{+\cdot}$, 73%), 95 ($[M—CH_2CO—CO]^{+\cdot}$, 93%), 82 (31%), 68 (93%) and 54 (53%). NMR ($CDCl_3$): 7.56 (d, 1H, $H_3$, $J$ = 1 Hz), 6.75 (d, 2H, $H_5$ and $H_6$, $J$ = 1 Hz) and 2.23δ (s, 3H, $CH_3$—CO).

[1′-*acetyl*-$^{14}$*C*]APBQ. [1-Acetyl-$^{14}$C]3HAA (5 mg, 0.03 mmole, 0.61 mCi/mmole) was diluted with unlabeled 3HAA (45 mg, 0.30 mmole), and the mixture was oxidized to the quinone according to the above procedure. Following recrystallization, a total of 11 mg of [$^{14}$C]APBQ was obtained (yield = 25%, sp. act = 0.06 mCi/mmole) which was judged to be >99% radiochemically pure by HPLC analysis.

2,5*DHAA*. This compound was synthesized as described previously [6].

[1-*Acetyl*-$^{14}$*C*]2,5*DHAA*. A solution containing [1′-acetyl-$^{14}$C]APBQ (1.1 mg, 0.007 mmole, 0.06 mCi/mmole) and unlabeled APBQ (2.8 mg, 0.017 mmole), dissolved in the minimum amount of ultrapure dimethyl sulfoxide (Alfa Products, Danvers, MA) was added to a solution of ascorbic acid (8.4 mg, 0.04 mmole) in water (4 ml). After stirring for 30 min at ambient temperature, sodium sulfate was added to the colorless solution, which was then extracted with ethyl acetate (3 × 5 ml). The pooled extracts were dried over sodium sulfate, evaporated under nitrogen, and the product was recrystallized from ethyl acetate. This yielded the radiolabeled 2,5DHAA (2.3 mg, 57%, 0.017 mCi/mmole) which was judged to be 99% radiochemically pure by HPLC analysis.

### *HPLC*

The radiochemical purities of synthetic [$^{14}$C]APBQ and [$^{14}$C]2,5DHAA were determined by HPLC analysis using a Waters model 6000 instrument, equipped with a Partisil 5 column (25 cm × 4.6 mm i.d., 5 μm; Whatman Inc., Clifton, NJ) and Waters model 440 UV detector ($\lambda$ = 254 nm). Samples were analyzed isocratically using ethyl acetate (1 ml/min) as mobile phase. The retention times of APBQ and 2,5DHAA under these conditions were 4.1 and 5.4 min respectively.

### *GLC*

Gas chromatography was carried out using a Hewlett–Packard model 5710A instrument, equipped with a fused silica capillary column (30 m × 0.32 mm i.d.) coated with the bonded stationary phase DB-5 (J & W Scientific, Ventura, CA). Helium (head pressure 20 psi) was employed as carried gas and compounds eluting from the GLC column were detected by means of a flame ionization detector (maintained at 250°) and recorded using a Hewlett–Packard model 3390A reporting integrator. Samples were injected using the splitless mode of operation (injector block temperature 250°) and "cold-trapped" on the column at 40°. After a delay of 30 sec, the temperature of the oven was raised rapidly to 100° and then programmed linearly to 250° at 8°/min. Identical GLC conditions to those described above were used in GC-MS work. Retention times were measured relative to a homologous series of *n*-alkanes co-injected with each sample and are expressed as methylene unit (MU) values.

### *Biological experiments*

Hepatic microsomes were prepared from adult male BALB/c mice that had been pretreated with phenobarbital (75 mg·kg$^{-1}$·day$^{-1}$, i.p., for 3 days). All incubations were carried out for 20 min in 25-ml Erlenmeyer flasks sealed with parafilm and placed in a shaking water bath (Forma Scientific model 2564) at 37°. Each incubation contained 3HAA, APBQ or 2,5DHAA (at the concentrations and specific activities indicated in the tables) and 6.0 mg of freshly prepared microsomal protein in 3.0 ml of phosphate buffer (final concentration, 0.17 M; pH 7.4). Some incubations also contained 6.0 mg of NADPH, as indicated. The method used for determination of covalent binding to protein is described elsewhere [9].

### *Analysis of metabolites*

Metabolites were identified by GC-MS on the basis of the both their GLC retention characteristics and their electron impact mass spectra. In all cases, the GC-MS properties of metabolites were shown to be identical to those of the respective reference compounds prepared by synthesis. Quantitative determinations of 2,5DHAA and 3,4DHAA in microsomal incubations were carried out by extraction of metabolites into ethyl acetate, conversion to TMS derivatives using BSTFA and analysis by GLC using 3′-methoxy-4′-hydroxyacetanilide as internal standard. Details of this assay procedure, which gives rise to two peaks (partially and fully silylated derivatives) for each of the above compounds, have been discussed previously [6]. The standard curve for 2,5DHAA was linear in the range 1–500 μg per incubation.

It was found that the same GLC assay procedure could also be employed to quantify APBQ present in incubation mixtures which did not contain NADPH or microsomes. Treatment of the quinone with BSTFA, however, gave incomplete conversion to the TMS derivative, and two peaks were obtained with MU values of 14.89 (underivatized APBQ) and 15.19 (APBQ TMS). Nevertheless, when the ratio of the sum of the areas for the quinone peaks to that of the internal standard was plotted against the amount of quinone present, a standard curve for APBQ was obtained which was linear in the range 15–400 μg per incubation.

## RESULTS

Two sets of experiments were carried out in this study. In the first, the metabolism and covalent binding properties of 3HAA in microsomal incubations were compared to those of the quinone, APBQ, while in the second, a similar comparison was made between 2,5DHAA and 3HAA.

The results of the first set of experiments are summarized in Table 1. [$^{14}$C]3HAA has been found to undergo metabolism to 2,5- and 3,4DHAA [6] and to bind covalently to microsomal protein. Both events were strongly NADPH dependent. Incubation of APBQ with microsomes led to the formation of 2,5DHAA. When NADPH was present, this reduction product accounted for the majority of the substrate consumed; when NADPH was omitted, however, substantially less 2,5DHAA was produced and several unidentified compounds of higher molecular weight, believed to be polymers of APBQ, were extracted from incubation mixtures (data not

Table 1. Covalent binding of [$^{14}$C]3HAA and [$^{14}$C]APBQ to microsomal protein

| Substrate | Addition | NADPH | Covalent binding to microsomes* (nmoles/mg) | Covalent binding to microsomes* (% Control) | Formation of 2,5DHAA† (nmoles/mg) | Formation of 3,4DHAA† (nmoles/mg) |
|---|---|---|---|---|---|---|
| 3HAA | – | + | 15.2 ± 0.2 | 100 | 43.2 ± 1.2 | 29.5 ± 0.6 |
| | | – | 4.2 ± 0.2 | 28 | ND‡ | ND |
| APBQ | – | + | 75.7 ± 1.9 | 497 | 459.8 ± 11.5 | ND |
| | | – | 167.8 ± 6.1 | 1102 | 121.3 ± 7.9 | ND |
| 3HAA | POBN | + | 18.2 ± 1.3 | 120 | 54.7 ± 5.0 | 28.1 ± 3.0 |
| | | – | 4.1 ± 0.3 | 27 | ND | ND |

* The covalent binding of [1-acetyl-$^{14}$C]3HAA (0.16 mCi/mmole, 1.0 mM) or [1'-acetyl-$^{14}$C]APBQ (0.06 mCi/mmole, 1.4 mM) to protein was determined in 20-min incubations with hepatic microsomes from phenobarbital-pretreated mice [9]. The spin-trapping agent POBN was employed at a concentration of 5.0 mM. All incubations were carried out in both the presence and absence of NADPH. Results are expressed as mean values ± S.E.M. (N = 3).

† The formation of 2,5DHAA and 3,4DHAA was determined in the same experiment as used for measurements of covalent binding.

‡ Not detected.

shown). The quinone was also found to bind extensively to microsomal protein in the presence of NADPH and, to an even higher degree, in the absence of this co-factor. Indeed, the covalent binding of APBQ to protein in the absence of NADPH was approximately eleven times that of 3HAA in the presence of NADPH. The spin-trapping agent, POBN, which was selected for its lack of inhibitory effects on cytochrome P-450 activity [10], had little effect on the microsomal metabolism or covalent binding of 3HAA in these experiments.

The results of the second series of experiments are summarized in Table 2 and show that, when NADPH was present, 2,5DHAA bound covalently to microsomal protein to a similar extent as 3HAA. When NADPH was omitted from incubation media, however, more than a 2-fold increase was obtained for the binding of 2,5DHAA. Moreover, when both NADPH and microsomes were omitted from incubations, the quinone APBQ was detected as an oxidation product of 2,5DHAA and accounted for approximately 19% of the initial substrate concentration at the end of a 20-min incubation (Figs. 1 and 2). In contrast, no quinone was detected when either NADPH or microsomal protein was included in incubation mixtures. Finally, neither superoxide dismutase (50 μg/ml) nor catalase (200 μg/ml) caused a significant decrease in the production of APBQ from 2,5DHAA in buffered aqueous solution, although a nitrogen atmosphere was found to decrease the rate of oxidation of 2,5DHAA in buffer by approximately 90% (data not shown).

## DISCUSSION

The results of this investigation further support the conclusions drawn from our earlier study that 3HAA is transformed by NADPH-dependent process in hepatic microsomes to the hydroquinone 2,5DHAA, the catechol 3,4DHAA, and a reactive, electrophilic intermediate which binds covalently to protein. In contrast, the quinone APBQ, which is formed by oxidation of 2,5DHAA, is a strongly electrophilic compound in its own right and binds readily to microsomal protein without undergoing metabolism. The extent to which the three com-

Table 2. Covalent binding of [$^{14}$C]2,5DHAA to microsomal protein and oxidation to APBQ

| Substrate | NADPH | Covalent binding to microsomes* (nmoles/incubation) | Covalent binding to microsomes* (% Control) | APBQ formation (nmoles/incubation) | APBQ formation (% Initial substrate) |
|---|---|---|---|---|---|
| 2,5DHAA | + | 33.4 ± 0.8 | 117 | ND† | ND |
| | – | 68.2 ± 1.7 | 239 | ND | ND |
| 3HAA | + | 28.5 ± 2.0 | 100 | ND | ND |
| | – | 4.6 ± 0.3 | 16 | ND | ND |
| 2,5DHAA | + | ‡ | | ND | ND |
| | – | ‡ | | 565 ± 41 | 19.1 |

*Covalent binding of [1-acetyl$^{14}$C]2,5DHAA (0.006 mCi/mmole, 1.0 mM) or [1-acetyl-$^{14}$C]3HAA (0.16 mCi/mmole, 1.0 mM) to protein was determined in 20-min incubations with hepatic microsomes from phenobarbital-pretreated mice [9]. All incubations were carried out in the presence and absence of NADPH. Results are expressed as mean values ± S.E.M. (N = 3).

† Not detected.

‡ No microsomes were present in these incubations.

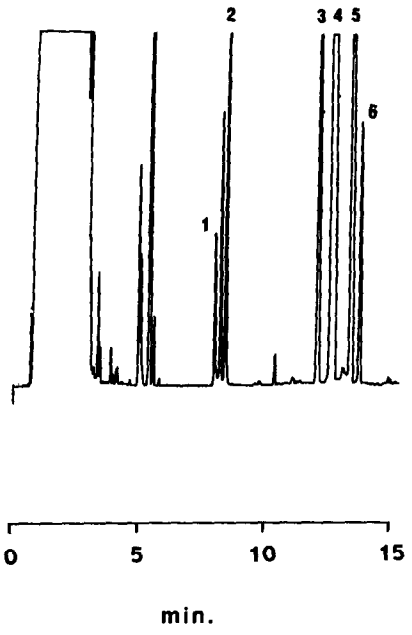


Fig. 1. GLC separation of products of incubation of 2,5DHAA. 2,5DHAA (1.0 mM in 0.25 M potassium phosphate buffer, pH 7.4) was incubated for 20 min at 37° in the absence of both microsomes and NADPH. Following acidification of the mixture, products were extracted, converted to TMS derivatives, and analyzed by capillary GLC as described in Materials and Methods. Numbered peaks correspond to the following compounds: 1, APBQ; 2, APBQ TMS; 3 and 6, *bis*- and *mono*-TMS derivatives, respectively, of 3'-methoxy 4'-hydroxyacetanilide (internal standard); 4 and 5, *tris*- and *bis*-TMS derivatives, respectively, of 2,5DHAA. Peaks showed here as "off-scale" were within the dynamic range of the recording integrator used for quanitative measurements.

pounds examined in this work become irreversibly bound to protein follows the order 3HAA < 2,5DHAA < APBQ. This finding is fully consistent with the mechanism for metabolic activation of 3HAA presented in Fig. 3 which depicts the quinone as being the reactive moiety binding to nucleophilic centres on protein. The effect of NADPH on decreasing the binding of radiolabel from both APBQ and 2,5DHAA is also consistent with this scheme and may be attributed to the reducing properties of the co-factor which shifts the APBQ ⇌ 2,5DHAA equilibrium to the hydroquinone form. The latter phenomenon is directly analogous to the situation with *N*-acetyl-*p*-benzoquinone imine, the reactive metabolite of acetaminophen, which is reduced very rapidly by NADPH to the parent drug and which binds to microsomal protein to a much lesser extent in the presence, as opposed to the absence, of an NADPH-regenerating system [11]. Further support for the scheme proposed in Fig. 3 may be derived from the experiment with POBN, a free radical spin-trapping agent (Table 1). Failure of POBN to decrease the covalent binding of radiolabel from 3HAA to protein or to alter the production of 2,5DHAA indicates that a semiquinone radical species, if formed as a transient intermediate in the oxidation of 3HAA to APBQ, probably does not contribute significantly to the covalent binding process. Interestingly, a slight *increase* in covalent binding of 3HAA was observed in the presence of POBN, although this increase was not statistically different from control values ($P < 0.05$). Such an increase could be indicative of a spin-exchange reaction occurring between POBN and the semiquinone radical, leading to enhanced production of APBQ and thus greater binding to protein.

With regard to the mechanism by which the quinone is formed from 2,5DHAA, it is clear from the experiment performed in the absence of both NADPH and microsomal protein that oxidation of this substituted hydroquinone may occur by purely chemical means. In related work, Greenlee *et al.* [12] have proposed a mechanism by which hydroquinone itself undergoes air-oxidation to benzoquinone, a putative reactive metabolite of benzene. Thus, it is possible that the conversion of 2,5DHAA to APBQ in microsomal preparations is not enzyme-mediated, although we cannot rule out alternative mechanisms by which oxidation of 2,5DHAA is catalyzed by NADPH-independent enzyme activity or is affected by a chemical interaction with the microsomal protein. Indeed, Sawahata and Neal [13] have speculated that the final steps in the conversion of hydroquinone to a reactive metabolite in rat liver microsomes are enzyme dependent and, furthermore, that they are mediated by an enzyme other than cytochrome P-450. Our efforts to detect quinone formation directly from 2,5DHAA in microsomal incubations that lacked NADPH were unsuccessful, possibly due to the high rate at which APBQ binds irreverisbly to protein and/or undergoes reduction back to 2,5DHAA.

In conclusion, the results of this investigation, together with those of our previous study [6], provide persuasive evidence that APBQ is a reactive metabolite of 3HAA which binds covalently to microsomal protein *in vitro*. Although the electrophilic character of quinones is well known [14], the involvement of these species as reactive intermediates in foreign compound metabolism has been recognized only in recent years. Thus, *p*-benzoquinone is believed to represent a toxic, electrophilic metabolite of both phenol and benzene [15, 16], reactive *p*-benzoquinone imine derivatives have been shown to result from the metabolic oxidation of drugs such as acetaminophen [11] and celiptium [17, 18], and quinone methides have been proposed as toxic metabolites of butylated hydroxytoluene [19, 20], diethylstilbestrol [21] and certain polycyclic aromatic hydrocarbons [22]. Much remains to be learned, however, about the covalent interactions occurring between these quinone derivatives and specific cellular macromolecules and of the role of these covalent binding processes in mediating the toxic effects of the parent compounds. In this context, it is of interest to note that, whereas 3HAA and its positional isomer acetaminophen evidently undergo metabolism to structurally similar *p*-benzoquinone and *p*-benzoquinone imine derivatives, the hepatotoxic properties of the two hydroxyacetanilides differ markedly. Studies on the selectivity of protein arylation by APBQ on the

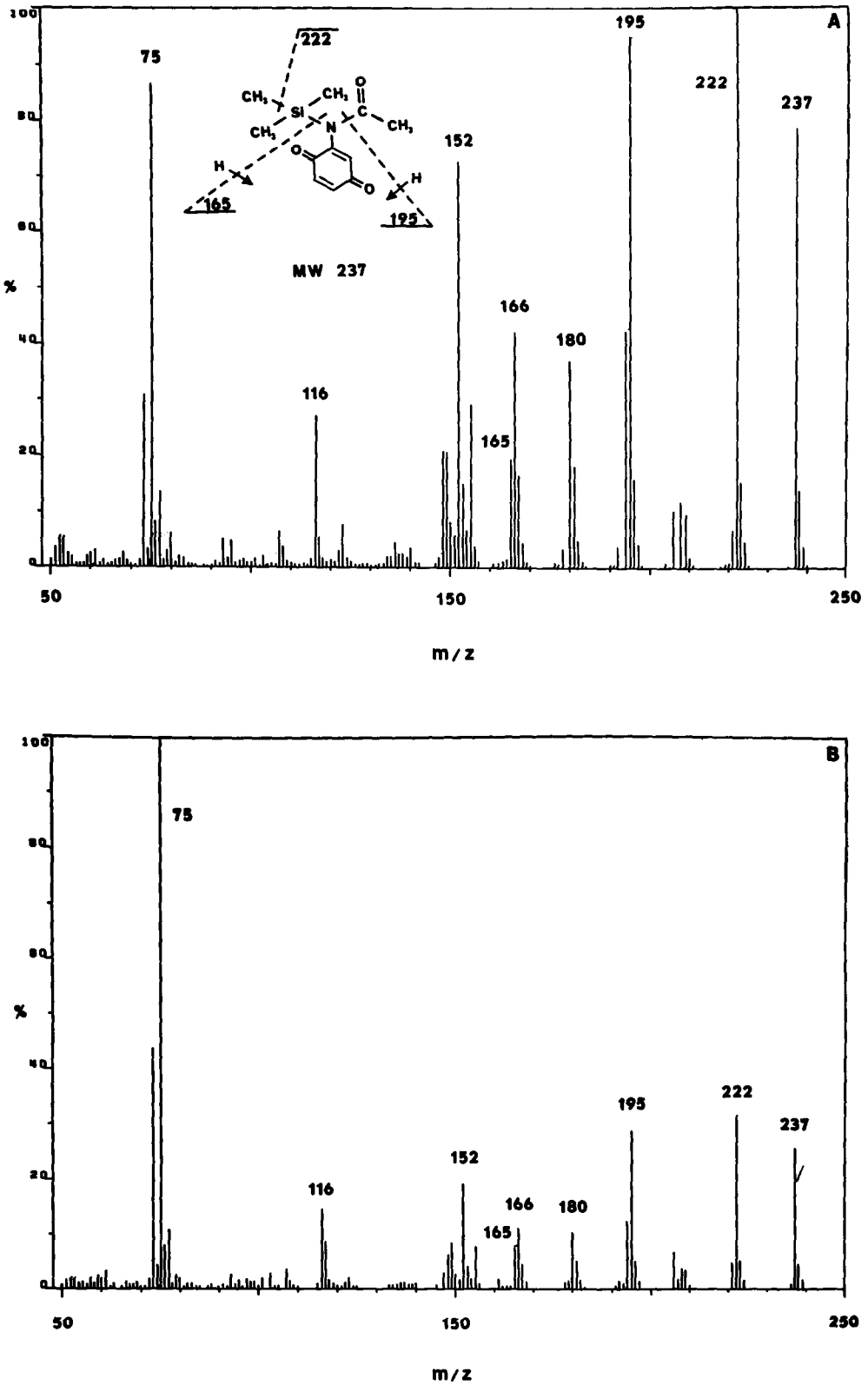


Fig. 2. Mass spectra of the APBQ TMS derivative. Electron impact (70 eV) mass spectra of the TMS derivative of (A) synthetic APBQ, and (B) APBQ extracted from an incubation of 2,5DHAA in buffer in the absence of both microsomes and NADPH (peak 2, Fig. 1). Both spectra were recorded under GC-MS conditions as described in Materials and Methods. The origin of certain key fragment ions is as indicated. The relative intensity of the ion at $m/z$ 75 was found to depend largely on the point at which the spectrum was recorded during elution of the derivative from the GLC column. The relative intensities of other ions in the spectrum, however, remained constant.

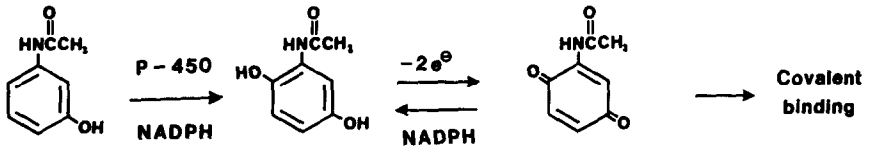


Fig. 3. Proposed scheme for the metabolic activation and covalent binding to protein of 3HAA.

one hand, and by *N*-acetyl-*p*-benzoquinone imine on the other, may provide an important insight into this intriguing toxicological issue.

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