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Identification of the Major Covalent Adduct Formed *in Vitro* and *in Vivo* between Acetaminophen and Mouse Liver Proteins

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SUMMARY

Improved analytical methodology has been developed for the structural characterization of covalently bound drug-protein adducts and has been applied to an investigation of the conjugates formed *in vivo* and *in vitro* between [^{14}C]acetaminophen and mouse liver proteins. The major adduct released by acid hydrolysis of hepatic protein samples, which accounted for approximately 70% of the bound radioactivity *in vivo* and *in vitro*, was identified as 3-cystein-*S*-yl-4-hydroxyaniline, a derivative whose structure reflects the predominance of acetaminophen thioether adducts in drug-modified proteins. It is concluded that the reactive, electrophilic metabolite of acetaminophen, which most likely is *N*-acetyl-*p*-benzoquinoneimine, binds with a high degree of selectivity to cysteinyl thiol groups on protein, formally in a Michael-type addition reaction. Cysteine residues thus represent primary target sites for arylation by the reactive metabolite of acetaminophen, and proteins rich in free thiols may be especially vulnerable to damage by this toxic intermediate.

INTRODUCTION

Acetaminophen (paracetamol, 4′-hydroxyacetanilide, *1*, Fig. 1) is a widely used nonprescription analgesic and antipyretic agent which is known to cause serious liver injury in both experimental animals and humans when taken in high doses (1, 2). The mechanism of acetaminophen-induced hepatic necrosis is a subject of considerable toxicological interest and is believed to be associated with the formation of a reactive, electrophilic metabolite of the drug NAPQI[2] (*2*, Fig. 1) which depletes cellular glutathione stores and binds covalently to tissue macromolecules (3, 4). Although the structure of the acetaminophen-glutathione conjugate (*3*, Fig. 1) has been firmly established by mass spectrometric (5) and NMR techniques (6), little is known about the nature of the covalent adduct(s) formed between NAPQI and protein. Indirect evidence suggests that arylation of proteins by this reactive metabolite may take place selectively at cysteinyl sulfhydryl groups (7, 8) to give thioether conjugates analogous to the acetaminophen-glutathione adduct. Recently, we have developed new analytical methodology for the degradation of drug-protein adducts with which we have been able to show directly that acetaminophen binds preferentially to the free thiol group in bovine serum albumin, a model protein which serves as an *in vitro* "trapping agent" for the electrophilic metabolite of acetaminophen (9).

In the work described in this report, our analytical methodology has been further developed for use in the direct structural characterization of drug-protein conjugates generated *in vivo* and has been applied to identify the major covalent adduct to hepatic microsomal and cytosolic proteins produced following the administration to mice of a hepatotoxic dose of acetaminophen. In addition, the nature of the acetaminophen-protein adduct formed *in vitro* during incubations of the drug with mouse liver microsomal preparations has been investigated by use of the same analytical protocol.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled acetaminophen was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was recrystallized from hot water before use. [*ring*-U-^{14}C]Acetaminophen (9.8 mCi mmol^{-1}) was obtained from Pathfinder Laboratories Inc. (St. Louis, MO) and was purified by HPLC as described previously (8). This radiolabeled material was diluted with unlabeled drug to give a final specific radioactivity of 1.92 mCi mmol^{-1} for *in vivo* studies and 0.80 mCi mmol^{-1} for *in vitro* experiments. A sample of the cysteine conjugate of acetaminophen (*4*) was synthesized by reaction of L-cysteine (Sigma Chemical Co., St. Louis, MO) with NAPQI (10), as described earlier (9). Ethyl chloroformate was purchased from Sigma, constant boiling HCl (Sequanal

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[2] The abbreviations used are: NAPQI, *N*-acetyl-*p*-benzoquinoneimine; *t*BDMS, *tert*-butyldimethylsilyl; MBSTFA, *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectrometry; MU, methylene unit; IP, intraperitoneal.

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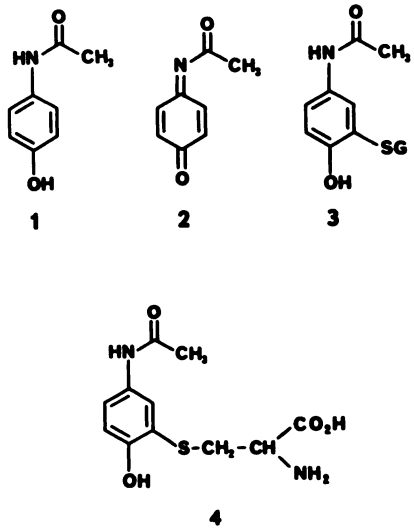

FIG. 1. *Structures of acetaminophen (1), NAPQI (2), the glutathione conjugate of acetaminophen (G, glutathion-S-yl residue) (3), and the cysteine conjugate of acetaminophen (4)*

grade) for protein hydrolysis was from Pierce Chemical Co. (Rockford, IL), and MBSTFA was obtained from Aldrich. Methanol and water for chromatography (HPLC grade) were purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ) and Burdick and Jackson Laboratories, Inc. (Muskegon, MI), respectively. Dichloromethane and glacial acetic acid (Baker) were of analytical grade.

HPLC procedures. Separations were carried out using a Beckman model 342 dual pump instrument, equipped with a Beckman model 160 fixed wavelength (λ = 254 nm) UV detector. Two HPLC systems were employed, as follows. System I was a Microsorb ODS semipreparative column (25 cm × 10 mm, 5 μm; Rainin Instruments, Berkeley, CA); isocratic elution was with methanol/glacial acetic acid/water (55:1:44 by volume) at a flow rate of 2 ml min^{-1}. System II was an Ultrasphere ODS analytical column (15 cm × 4.6 mm, 5 μm; Rainin Instruments); solvent A was methanol/glacial acetic acid/water (15:1:84 by volume); solvent B was methanol/glacial acetic acid/water (50:1:49 by volume); elution was with a linear gradient from 0–100% solvent B over 20 min at a flow rate of 1 ml min^{-1}, followed by isocratic elution at 100% solvent B for 10 min. Return to solvent A was carried out with a reverse gradient over 10 min, following which the column was equilibrated for at least 30 min before injection of the next sample.

Biological experiments. Adult male BALB/c mice (approximately 25 g) were obtained from Simonsen (Gilroy, CA) and were pretreated for 3 days with sodium phenobarbital (75 mg kg^{-1} per day, IP) before use. Metabolic studies were conducted 24 hr after the last phenobarbital injection.

In vivo experiments were carried out as follows. Four animals were dosed with [^{14}C]acetaminophen (200 mg kg^{-1}, administered IP as a 6 mg ml^{-1} solution in 0.9% NaCl) and were sacrificed by decapitation 4 hr after dosing. The livers were homogenized individually in distilled water (6 ml), microsomal and cytosolic fractions from each homogenate were obtained by ultracentrifugation (8), and the microsomal pellets were resuspended in distilled water (5 ml). One-half of each microsomal and cytosolic sample was taken for determination of radioactivity covalently bound to protein (8). The remaining microsomal suspensions were pooled, as were the cytosolic fractions. In both sets, the protein was precipitated by addition of trichloroacetic acid (3 M, 2 ml), and the precipitate was washed in turn with 5-ml portions of trichloroacetic acid (0.63 M), ethanol/diethyl ether (3:1, v/v), and diethyl ether. The final protein pellets were air-dried overnight.

For the *in vitro* experiments, hepatic microsomes were prepared and incubated with [^{14}C]acetaminophen (1 mM) as described previously (8). The microsomes were then re-isolated by centrifugation at 100,000 × *g* and suspended in 45 ml of 0.1 M phosphate buffer, pH 7.4, at a concentration of 3 mg of protein ml^{-1}. The protein was precipitated by the addition of trichloroacetic acid (3 M, 5 ml) and collected by brief centrifugation (1,000 × *g*). This final protein pellet was washed as described above for the *in vivo* experiment and was air-dried.

Degradation of acetaminophen-protein adducts. The general procedure is summarized in Fig. 2 and represents an extension of an analytical protocol developed recently in our laboratories for the identification of the covalent adduct formed *in vitro* between acetaminophen and bovine serum albumin (9). This improved methodology was established from preliminary studies with the synthetic acetaminophen cysteine conjugate (*4*), which served as a model drug-amino acid adduct; the products of the various reactions with *4* were isolated by HPLC (system II) and were characterized by direct insertion mass spectrometry.

Samples of microsomal or cytosolic protein were dissolved in constant-boiling HCl and heated under vacuum in a sealed glass vial at 120° for 24 hr. The resulting solutions were evaporated to dryness (Rotavapor, 45°); the residues were taken up in pH 6 phosphate buffer (3 ml) and treated with acetic anhydride (0.1 ml). After stirring at ambient temperature for 10 min, aliquots were taken for measurement of radioactivity and NaOH (5 M) was added to raise the pH to 12. These solutions were then allowed to stand for a further 10 min before the pH was adjusted to 9 and ethyl chloroformate (0.05 ml) was added. After 30 min at room temperature, the reaction products were washed with dichloromethane (10 ml) and portions of the organic phases were taken for measurements of radioactivity. The aqueous layers were acidified to pH 2 with HCl (12 M) and extracted with ethyl acetate (3 × 10 ml). The combined ethyl acetate extracts were evaporated to dryness *in vacuo* and the residues were dissolved in methanol/acetic acid/water (55:1:44 by volume, 1.0 ml) for purification by HPLC (system I). Samples were injected in several portions, and fractions were collected at 30-sec intervals from 4–14 min. Aliquots of each column fraction were removed for liquid scintillation counting and those fractions containing appreciable amounts of radioactivity were evaporated to dryness and treated with MBSTFA/dimethylformamide (1:1, v/v, 50 μl) at 120° for 30 min. These final, derivatized samples were analyzed directly by GLC and GC-MS.

GLC and GC-MS procedures. GLC analyses were carried out on a Hewlett-Packard model 5710A gas chromatograph, modified for operation with capillary columns, and equipped with a split/splitless injection system (held at 250°), flame ionization detector (also maintained at 250°), and Hewlett-Packard model 3390A reporting integrator. A fused silica capillary column (60 m × 0.32 mm i.d.), coated with the bonded stationary phase DB-5 (J and W Scientific, Ventura, CA), was employed with helium (head pressure, 20 p.s.i.) as both carrier gas and detector make-up gas. Samples were injected using the splitless mode of operation and "cold-trapped" on the column at 40°. After a period of 30 sec, the column oven temperature was raised rapidly to 240° and, 5 min after injection, was programmed linearly at 3° min^{-1} to 300°. Retention times were measured relative to a homologous series of *n*-alkanes co-injected with each sample and are expressed as MU values.

GC-MS analyses were performed on a VG 70-70H double focusing mass spectrometer, equipped with a Hewlett-Packard model 5710A gas chromatograph, with on-line connection to a VG model 2035 data system. The mass spectrometer was operated in the electron impact mode (electron energy, 70 eV), with an ion source temperature of 200°, an emission current of 200 μamp, and an accelerating potential of 4 kV. The GLC column and operating conditions were the same as those noted above and mass spectra were recorded at a scan rate of 1 sec decade^{-1}.

Mass spectrometry. Mass spectra of reference compounds and intermediates obtained during the method development phase of the work were recorded on the same instrument as used for GC-MS studies. In this case, however, samples were introduced by the direct insertion technique using a Vespel-tipped probe. The mass spectrometer was operated in the chemical ionization mode (methane as reagent gas at a

source pressure of 0.5 torr; source temperature, 200°) and spectra were taken at a scan rate of 5 sec decade^{-1}.

RESULTS

Our earlier model studies on the structure of the covalent adduct formed *in vitro* between acetaminophen and bovine serum albumin demonstrated that the single free cysteine residue in this protein is the primary site of arylation (9). Based on this finding, attempts were made to extend the analytical protocol for work with complex, heterogeneous protein samples by incorporating capillary GLC as a final separation step. The rationale for including GLC in the analytical scheme was 2-fold. First, it was found in pilot experiments (not reported here) that acid hydrolysis of protein from the livers of mice exposed to hepatotoxic doses of [^{14}C] acetaminophen released one major drug-amino acid adduct which had the liquid chromatographic properties of the cysteine conjugate *5*. However, HPLC procedures alone were not adequate for the purification of this conjugate from the complex protein hydrolysates to a degree suitable for mass spectrometric identification. It was considered that the combination of HPLC and GLC techniques would be a more effective approach to the characterization of this adduct. Second, inclusion of a final GLC separation step would permit the use of GC-MS techniques for positive identification of the isolated adduct, thereby replacing the less sensitive and less specific direct insertion approach employed in our previous studies.

Method development was therefore directed towards the preparation of a chemically stable, volatile derivative of *5*, the anticipated major hydrolysis product of acetaminophen-protein covalent adducts, which would be suitable for gas-phase analysis. This work, which utilized the synthetic acetaminophen-cysteine conjugate (*4*) as a model compound for hydrolysis and derivatization studies, indicated that compounds bearing the aromatic *N*-acetyl moiety were suitable for GLC and GC-MS purposes, whereas the corresponding *N*-ethoxycarbonyl derivatives (such as that employed in our earlier protocol) were thermally unstable and decomposed upon injection into the gas chromatograph. Unfortunately, the acetyl group in covalently bound acetaminophen is removed quantitatively during the initial acid hydrolysis step and a means was sought, therefore, to replace it at an early stage of the analytical sequence. Acetylation of the aminophenol-cysteine conjugate (*5*) with excess acetic anhydride in buffered aqueous media at pH values of 6–7 gave rise to two products with retention times of 5.8 and 13.8 min in HPLC system II; no starting material (retention time, 3.6 min) remained after 10-min reaction at ambient temperature. (The aminophenol conjugate *5* is stable only under acidic conditions and decomposes rapidly at pH values greater than 7.) The products of acetylation were isolated, converted to their methyl esters by brief exposure to methanolic HCl, and analyzed by chemical ionization mass spectrometry. The product with the shorter retention time on HPLC proved to be the *N,N'*-bisacetyl derivative (*6*), whose mass spectrum exhibited a prominent MH$^+$ species at *m/z* 327, while the product with the longer retention time was found to be the fully derivatized *N,N',O,*-trisacetyl species *7* (MH$^+$ at *m/z* 369). Acetylation conditions could not be found, however, which yielded only a single product. Consequently, the mixture of bis- and trisacetyl derivatives was made alkaline (pH 12) for a brief period (10 min) in order to hydrolyze selectively the *O*-acetyl function and convert the mixture into the bisacetyl species *6* (Fig. 2). This latter compound (the mercapturic acid of acetaminophen) was then further derivatized by reaction in aqueous solution with ethyl chloroformate (9) and the *O*-ethoxycarbonyl product *8* was extracted into ethyl acetate and purified by HPLC (retention time of 18.2 min in system II). The identity of *8* (as its methyl ester) was verified by chemical ionization mass spectrometric analysis, which gave the following ions: *m/z* 399 (MH$^+$), 357 ([MH—CH_2CO]$^+$), 328 ([357—C_2H_5]$^{+\cdot}$), 284 ([357—$CO_2C_2H_5$]$^{+\cdot}$),256([MH—CH_2═$C(CO_2CH_3)NHCOCH_3$]$^+$),

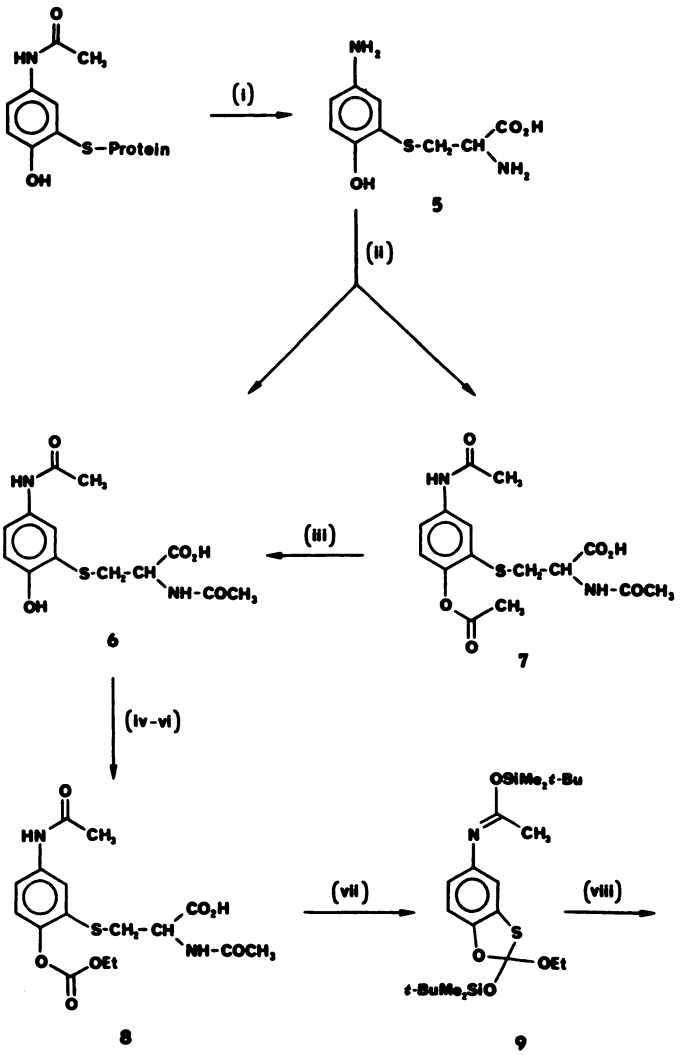

FIG. 2. *Scheme for the analysis of protein-bound thioether conjugates of acetaminophen*

Step i, acid hydrolysis; step ii, acetylation; step iii, hydrolysis of the *O*-acetyl group in *7*; step iv, reaction with ethyl chloroformate; step v, extraction into ethyl acetate; step vi, purification by reverse phase HPLC; step vii, reaction with MBSTFA; step viii, analysis of product 9 by GLC and GC—MS. For details, see Experimental Procedures.

210 ([256—C_2H_5OH]$^{+\cdot}$), 144 (complementary ion to 256), and 102 [$^+CH_2$—$CH(CO_2CH_3)NH_2$].

The final reaction in the scheme shown in Fig. 2 involved derivatization of the acid *8* for GLC and GC-MS analysis. Investigation of a number of esterification procedures revealed that a novel reaction takes place with the silylating agent MBSTFA, in which the S-CH_2 bond of the cysteinyl moiety is cleaved and a bis-*t*BDMS derivative, to which we tentatively assign the orthoester structure *9*, is formed. This reaction, which requires the ethoxycarbonyl substituent on the phenolic oxygen of the acetaminophen ring system, probably proceeds by way of a cyclic five-membered sulfonium ion formed by attack of the sulfur atom on the neighboring carbonyl group; the latter intermediate would be predisposed towards loss of the major part of the cysteinyl moiety in a retro-Michael reaction to yield the observed product. This derivatization step, which gave the *t*BDMS ether *9* in apparently quantitative yield, proved to be a valuable modification of the earlier protocol in that a volatile derivative of the adduct was produced which exhibited excellent GLC and GC-MS characteristics.

Application of this modified analytical protocol to the study of acetaminophen-protein covalent adducts was made first to microsomal and cytosolic protein fractions from the liver of mice given a hepatotoxic dose (200 mg kg^{-1}) of radiolabeled drug. Four hours after drug administration, covalent binding of acetaminophen to hepatic microsomal and cytosolic protein was found to be 0.56 ± 0.14 (n = 4) and 0.90 ± 0.18 nmol mg^{-1} (n = 4), respectively. These figures are in good agreement with published values for the covalent binding of this dose of acetaminophen to mouse liver proteins (11).

Following acid hydrolysis of the acetaminophen-microsomal protein adduct and formation of the *N,N'*-bisacetyl, *O*-ethoxycarbonyl derivative *8* (steps i–iv, Fig. 2), approximately 80% of the radioactivity present in the initial protein sample was recovered by extraction into ethyl acetate (step v, Fig. 2), while a further 5 and 10% were found in the dichloromethane wash and in the aqueous phase after pH 2 extraction, respectively. Analysis of this derivatized protein hydrolysate by HPLC (system I) revealed a complex profile of UV-absorbing components (Fig. 3, upper trace). Recovery of radioactivity from the column was essentially quantitative with 72% of the counts eluting in fractions 12 and 13 and a further 17% in fraction 8 (Fig. 3, lower trace). The major peak had exactly the same retention volume as the derivative produced from an authentic sample of 3-cysteinylacetaminophen (*4*) which had been subjected to the same analytical procedure (Fig. 3, center trace). The minor peak eluted at the retention volume of the trisacetyl derivative *7*, present in the sample as a residue from incomplete hydrolysis at an earlier step in the analytical protocol (Fig. 2, step iii). When the material contained in column fractions 12 and 13 was pooled, treated with MBSTFA, and analyzed by GLC, the gas chromatogram reproduced in Fig. 4 was obtained. Despite the complexity of this "purified" sample, a peak with the same retention index (MU = 26.5) as the reference silyl derivative *9* eluted at 19.5 min. Analysis of this compo-

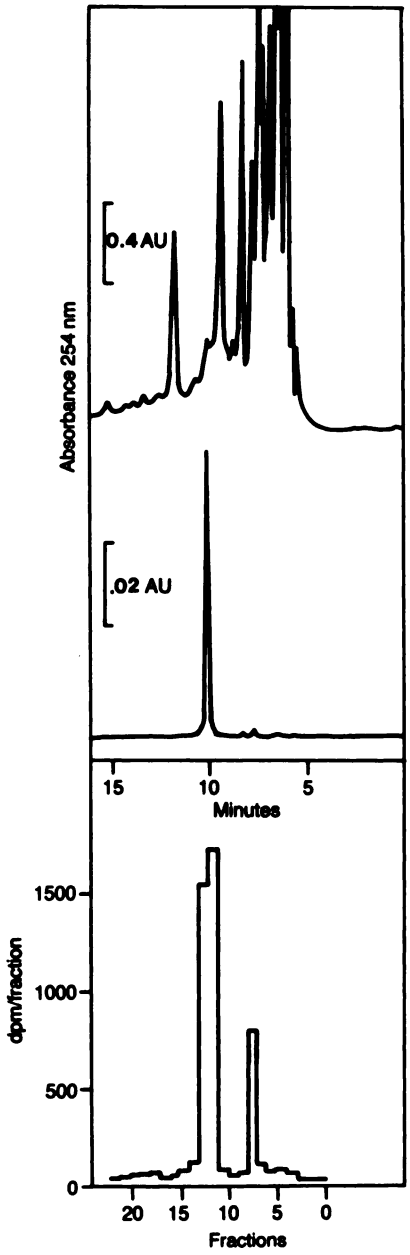

FIG. 3. *Reverse phase HPLC analysis of the derivatized (N,N'-bisacetyl, O-ethoxycarbonyl) acid hydrolysate from the microsomal fraction of mouse liver obtained 4 hr after an IP dose of [^{14}C]acetaminophen (200 mg kg^{-1})*

Upper frame, profile of UV-absorbing compounds eluting from the column; center frame, chromatography of the derivatized product (*8*) obtained from an acid hydrolysate of authentic 3-cystein-*S*-yl-acetaminophen; lower frame: profile of radioactivity eluting from the column. *AU*, absorbance units.

nent by GC-MS yielded the mass spectrum shown in Fig. 5B, which was essentially identical to that of the reference thioether derivative *9* (Fig. 5A). Salient features of this mass spectrum include the $M^{+\cdot}$ ion at m/z 483 and fragment ions at m/z 468 ([M—CH_3]$^+$), 438 ([M—OC_2H_5]$^+$), 426 ([M—*t*Bu]$^+$), 352 ([M—*t*$BuMe_2SiO$]$^+$), 296 ([352—C_4H_6]$^+$), and 222 ([352—*t*$BuMe_2SiO$+H]$^+$). Moreover, based on analysis of known amounts of the synthetic derivative *9* as an external standard, the detec-

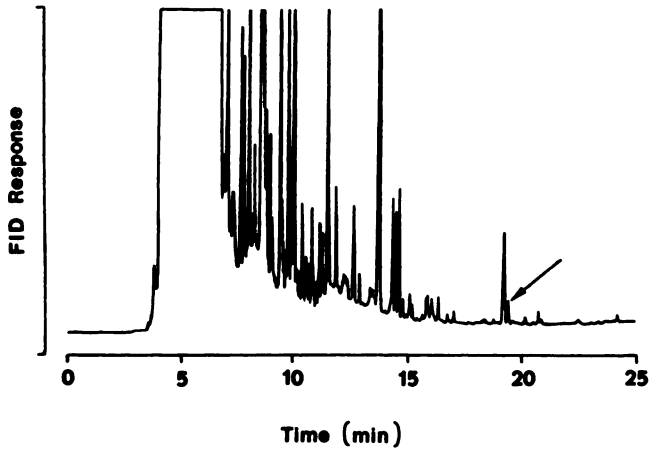

FIG. 4. *Gas chromatographic separation of tBDMS ether derivatives obtained from pooled radioactive fractions 12 and 13 collected from the HPLC analysis depicted in Fig. 3*

The peak with the same retention index as the authentic *t*BDMS derivative *9* is indicated by an arrow. *FID*, flame ionization detector.

tor response on GLC and the intensity of the mass spectrum obtained on GC-MS analysis of the biological material were consistent with *all* of the injected sample being in the form of derivative *9*. It may be concluded, therefore, that the major covalent adduct of acetaminophen to microsomal protein *in vivo* is a 3-cysteinyl thioether conjugate, analogous to that formed *in vitro* with bovine serum albumin (9). It should be pointed out that, although the acid hydrolysis procedure used in this work removes the acetamide sidechain of acetaminophen, it is known from studies with specifically labeled radioactive analogs of acetaminophen that the bulk of the covalently bound material both *in vitro* (4) and *in vivo* (11) retains the acetyl group. Thus, the principal protein adducts must be 3-cystein-*S*-yl conjugates of acetaminophen itself. Analysis of the radioactivity measurements cited above indicate that approximately 72% of the drug which is bound covalently to microsomal protein is in the form of this one thioether conjugate.

Degradation of the corresponding acetaminophen-cytosolic protein adduct gave results which were very similar to those just described and indicated that the bulk of the radioactivity released by acid hydrolysis of this modified protein also co-chromatographed on HPLC with the authentic cysteine conjugate of acetaminophen (Fig. 6). The identity of the principal radioactive adduct (fraction 13, Fig. 6) was then confirmed by GLC and GC-MS analysis to be the same as that obtained from the microsomal protein fraction (data not shown).

In the final experiment, where acetaminophen was incubated with hepatic micromsomes from phenobarbital-pretreated mice, covalent binding to protein was found to be 3.19 nmol mg^{-1}. Hydrolysis of this material, followed by acetylation and ethoxycarbonyl formation, yielded a product whose HPLC profile is shown in Fig. 7. The pattern of radioactive peaks eluting from the column was very similar to that recorded from each of the *in vivo* samples, the major component (fractions 12 and 13) again co-chromatographing with the synthetic cysteine conjugate derivative. When this component was silylated and analyzed by GLC and GC-MS, it was shown to be identical to the derivative *9* obtained from the previous experiments.

DISCUSSION

The improved analytical methodology developed in this work, which takes advantage of the differential reactivities in aqueous media of —NH_2, phenolic —OH, and —CO_2H groups and which exploits a novel silylation reaction for GC—MS studies, made it possible to demonstrate directly that when phenobarbital-pretreated mice are administered a hepatotoxic dose of acetaminophen, the principal covalent adduct to liver microsomal and cytosolic proteins in a thioether conjugate of the drug with cysteine sulfhydryl groups. Moreover, when the drug is incubated *in vitro* with mouse hepatic microsomes, the same adduct predominates. Judging from the HPLC profiles of radioactivity obtained from the various protein hydrolysates (Figs. 3, 6, and 7), it appears that some 72% of the bound acetaminophen in each case is in the form of this one thioether conjugate. Also, since the analytical protocol developed in this work was designed to have general applicability to drug-amino acid conjugates of a variety of structural types, other acetaminophen-protein adducts, had they been present, should have been detected by HPLC. It may be concluded, therefore, that covalent binding of acetaminophen to liver protein exhibits a remarkably high degree of selectivity for —SH groups, which thus represent primary target sites for attack by the reactive metabolite of the drug.

The structure of the isolated acetaminophen-cysteine adduct, in which the sulfur atom is attached to the C-3′ position of the drug moiety, is directly analogous to the conjugate formed *in vitro* by chemical reaction between cysteine and synthetic NAPQI (9) and suggests that the protein adducts may be formed by a similar reaction with NAPQI. On the other hand, the *N*-acetyl-*p*-benzosemiquinoneimine radical may also be formed by enzymatic oxidation of acetaminophen (12, 13) and might be expected to give rise to protein thioether conjugates of the type identified in this study. However, based on the absence of radioactivity covalently bound to the hepatic lipids of animals given toxic doses of radiolabeled acetaminophen, it has been proposed that such a free radical does not play a quantitatively significant role in the chemical interaction of acetaminophen-reactive metabolites with hepatic tissue macromolecules (14).

In a recent paper, Miller and Jollow (15) examined the effect of L-ascorbic acid on acetaminophen-induced hepatotoxicity and covalent binding in the hamster. Although ascorbate inhibited the covalent binding *in vitro* [presumably by reducing NAPQI back to acetaminophen (4)], it failed to protect against hepatic necrosis and did not suppress covalent binding to liver protein *in vivo* when given immediately after acetaminophen, despite the fact that reduced ascorbate levels in liver were markedly elevated. Furthermore, covalent binding of acetaminophen in isolated hamster hepatocytes was found to be partially inhibited by ascorbate, in contrast to both the *in vitro* and *in vivo* results. Based on these findings,

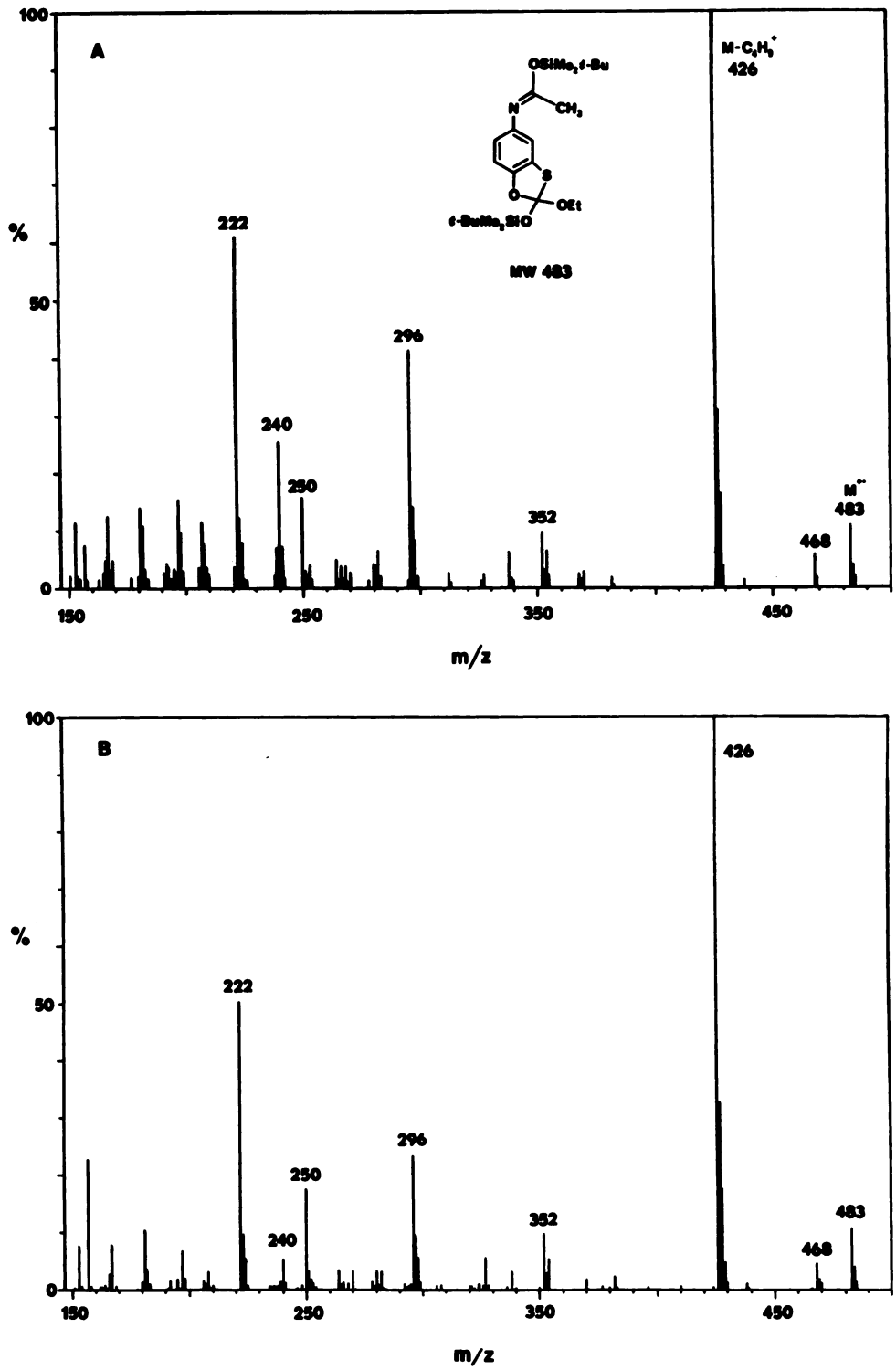

FIG. 5. *Electron impact mass spectra*

Recordings were made under GC-MS conditions of: (A) the final product (*9*) obtained by subjecting an authentic sample of the acetaminophen-cysteine conjugate (*4*) to the scheme shown in Fig. 2, and (B) the product obtained by reacting the biological isolate (fractions 12 and 13, Fig. 3) with MBSTFA and which gave rise to the indicated peak in Fig. 4.

the authors proposed that the reactive metabolite of acetaminophen formed *in vitro* differs from that produced *in vivo* in terms of its kinetic handling or its chemical nature. While the results of our studies do not completely exclude this possibility, they do demonstrate that the HPLC profile of acetaminophen-protein covalent adducts formed in the mouse *in vivo* is the same as that obtained from *in vitro* experiments. Considered collectively, the results of our work and those discussed above strongly support the contention that covalent binding of acetaminophen to liver tissue results from cytochrome P-450-mediated oxidation of the drug to

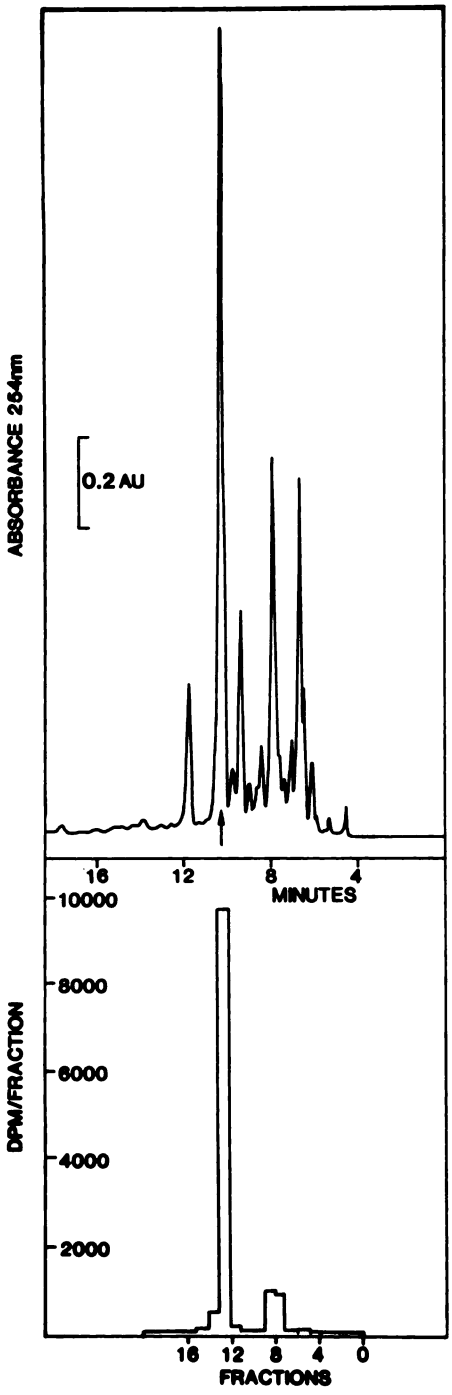

FIG. 6. *Reverse phase HPLC analysis of the derivatized (N,N'-bisacetyl, O-ethoxycarbonyl) acid hydrolysate from the cytosolic fraction of mouse liver obtained 4 hr after an IP dose of [^{14}C]acetaminophen (200 mg kg^{-1})*

Upper frame, profile of UV-absorbing compounds eluting from the column after spiking the biological isolate with an authentic sample of *8* as a retention time marker (the peak due to added *8* is indicated by an arrow); lower frame, profile of radioactivity eluting from the column.

NAPQI, a highly electrophilic intermediate which reacts preferentially with —SH groups (3, 4). Whether this binding of NAPQI to protein thiols occurs by a direct Michael addition reaction, or by initial *ipso* attack by sulfur at the C-1 imine or C-4 carbonyl carbons of

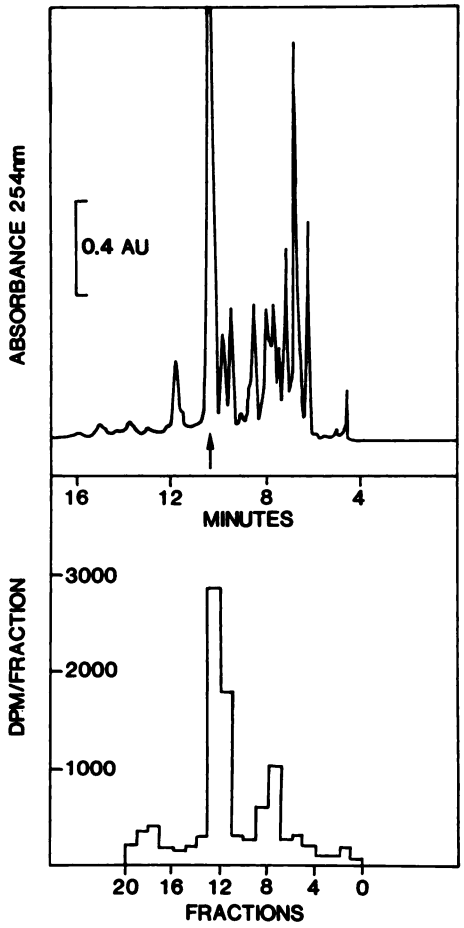

FIG. 7. *Reverse phase HPLC analysis of the derivatized (N,N'-bisacetyl, O-ethoxycarbonyl) acid hydrolysate from an in vitro incubation of [^{14}C]acetaminophen with mouse liver microsomes*

Upper frame, profile of UV-absorbing compounds eluting from the column after spiking the biological isolate with an authentic sample of *8* as a retention time marker (the peak due to added *8* is indicated by an arrow); lower frame, profile of radioactivity eluting from the column.

NAPQI (16) and subsequent rearrangement to the 3-thioether conjugate, remains to be established. In any event, it is clear that proteins which are rich in free thiol functions will be especially vulnerable to arylation damage by NAPQI generated intracellularly, a phenomenon which may be of importance in the pathology of acetaminophen-induced liver injury.

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