# Characterization of Chlorinated Adducts of Hemoglobin and Albumin Following Administration of Pentachlorophenol to Rats

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Five cysteinyl adducts (including one with multiple isomeric forms) of hemoglobin (Hb) and albumin (Alb) have been characterized in the blood of Sprague-Dawley rats following administration of pentachlorophenol (PCP). Three of these adducts were formed by multiple substitution reactions of tetrachloro-1,4-benzoquinone ($Cl_4$-1,4-BQ) and its products, and two arose from reactions of tetrachloro-1,4-benzosemiquinone ($Cl_4$-1,4-SQ) and tetrachloro-1,2-benzosemiquinone ($Cl_4$-1,2-SQ). Adducts of tetrachloro-1,2-benzoquinone ($Cl_4$-1,2-BQ) were not observed. Regarding adducts of $Cl_4$-1,4-BQ and its products, specific structures were assigned to monosubstituted, disubstituted, and trisubstituted adducts of Hb and Alb following modification of rat blood with $Cl_4$-1,4-BQ (0−45 $\mu$M) in vitro and after metabolism of PCP (0−40 mg/kg body weight) in Sprague-Dawley rats, in vivo. The formation of all adducts was linear over the ranges tested, with Alb adducts being more abundant than Hb adducts. The levels of the adducts measured were in the following order: monosubstituted > disubstituted > trisubstituted. The observation that $Cl_4$-1,4-BQ can produce multisubstituted adducts with proteins suggests that protein−protein cross links may be formed, with inherent toxicological implications. Regarding adducts of the semiquinones (detected only in vivo), linear production of Hb and Alb adducts was observed with increasing dosage of PCP for adducts of both $Cl_4$-1,4-SQ and $Cl_4$-1,2-SQ. Higher levels of the semiquinone adducts were observed in Hb than in Alb, in contrast to the results with the quinone adducts. In a separate in vivo experiment (20 mg PCP/kg body weight), where animals were sacrificed at intervals up to 336 h postadministration, adducts were eliminated at rates which were comparable among the different adducts of a given protein.

## Introduction

Pentachlorophenol (PCP)[1] is a widely used biocide which has become ubiquitous in environmental samples and which is commonly observed in human urine (*1*). Given such widespread distribution of PCP in the environment, and with evidence that PCP has caused cancers in rodent bioassays (*2*,*3*), it is important to understand the disposition of the reactive, and presumably toxic, metabolites of PCP in mammalian systems.

Much of the speculation involving the genotoxicity of PCP has focused upon quinone and semiquinone metabolites, which are illustrated in Figure 1. PCP is oxidatively dechlorinated to produce tetrachlorohydroquinone ($Cl_4$HQ) and tetrachlorocatechol ($Cl_4$CAT) by liver enzymes from rats and humans in vitro (*4*,*5*) and by rodents in vivo (*6*−*9*). $Cl_4$HQ and $Cl_4$CAT can be further oxidized to tetrachloro-1,4-benzoquinone ($Cl_4$-1,4-BQ) and tetrachloro-1,2-benzoquinone ($Cl_4$-1,2-BQ), respectively, via the corresponding semiquinones. The presence of tetrachloro-1,4-benzosemiquinone ($Cl_4$-1,4-SQ) has been confirmed by ESR spectroscopy, in an incubation mixture containing $Cl_4$HQ and oxygen, with an estimated lifetime of 5 s (*10*,*11*). Cytochrome P450-mediated direct oxidation of pentahalogenated phenols to tetrahalogenated benzoquinones and tetrahalogenated benzosemiquinones has also been proposed, via elimination of either a halide anion or a halide radical, respectively (*12*).

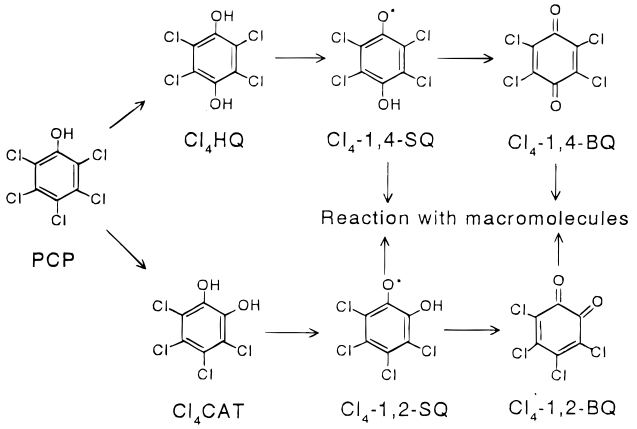


**Figure 1.** Metabolic pathways of PCP leading to the formation of active electrophiles.

Since these quinone and semiquinone species are strong electrophiles, they can react with macromolecules to form adducts. Covalent binding of $Cl_4$-1,4-BQ to rat

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[1] Abbreviations: Alb, albumin; $Cl_2$BQ, dichlorobenzoquinone; $Cl_2$-HQ, dichlorohydroquinone; DTPA, diethylenetriaminepentaacetic acid; $Cl_2$BQ-$Y_2$, disubstituted protein adduct of tetrachloro-1,4-benzoquinone; EI, electron ionization; Hb, hemoglobin; HFBI, *N*-heptafluorobutyrylimidazole; MTBE, methyl *tert*-butyl ether; ClBQ, monochlorobenzoquinone; ClHQ, monochlorohydroquinone; $Cl_3$BQ-Y, monosubstituted protein adduct of tetrachloro-1,4-benzoquinone; NICI, negative ion chemical ionization; PCP, pentachlorophenol; $Cl_4$-1,2-SQ-Y, protein adduct of tetrachloro-1,2-benzosemiquinone; $Cl_4$-1,4-SQ-Y, protein adduct of tetrachloro-1,4-benzosemiquinone; $Cl_4$-1,2-BQ, tetrachloro-1,2-benzoquinone; $Cl_4$-1,4-BQ, tetrachloro-1,4-benzoquinone; $Cl_4$-1,2-SQ, tetrachloro-1,2-benzosemiquinone; $Cl_4$-1,4-SQ, tetrachloro-1,4-benzosemiquinone; $Cl_4$CAT, tetrachlorocatechol; $Cl_4$HQ, tetrachlorohydroquinone; TCP, tetrachlorophenol; $Cl_3$BQ, trichlorobenzoquinone; $Cl_3$HQ, trichlorohydroquinone; ClBQ-$Y_3$, trisubstituted protein adduct of tetrachloro-1,4-benzoquinone.

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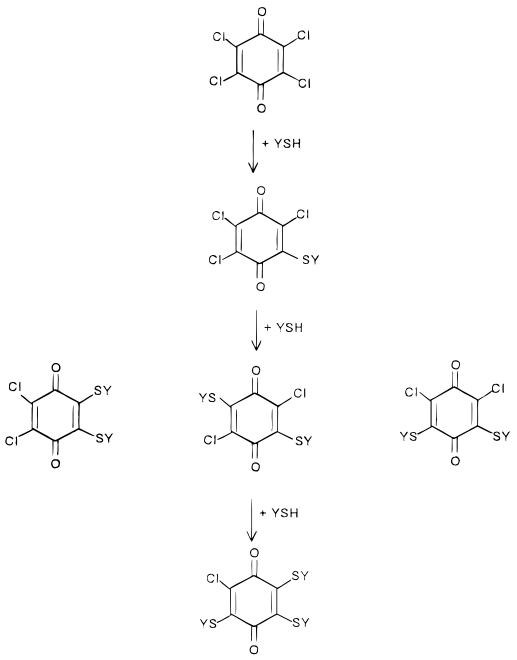


**Figure 2.** Possible pathways for the formation of multisubstituted cysteinyl adducts of $Cl_4$-1,4-BQ in proteins. Note that YSH could be a protein and/or a nonprotein thiol such as GSH.

microsomal proteins and DNA (*13*) and to calf thymus DNA (10) in vitro and to rat blood proteins in vitro and in vivo (*14*) has been reported. Macromolecular adducts of $Cl_4$-1,4-BQ with cysteinyl residues of the blood proteins albumin (Alb) and hemoglobin (Hb) have been characterized in vitro following incubation of rat blood with $Cl_4$-1,4-BQ and in vivo following administration of PCP to rats (*14*). These adducts resulted from reactions of $Cl_4$-1,4-BQ with sulfhydryl groups of cysteinyl residues, in which a chlorine atom was eliminated from the aromatic ring (*14*).

It has been shown that $Cl_4$-1,4-BQ retains its oxidized quinone structure after reaction with one molecule of GSH and continues to react with additional GSH molecules until all four chlorine atoms have been substituted (*15*). Three GSH conjugates of $Cl_4$-1,4-BQ have been characterized (*15*). The observation that these GSH-derived quinone thioethers undergo further reactions with GSH suggests that protein-derived analogues will behave in a similar fashion through reactions with accessible sulfhydryls. Hence, one would expect $Cl_4$-1,4-BQ and $Cl_4$-1,2-BQ to participate in multiple reactions with sulfhydryls leading to the formation of both protein−protein cross links and/or to protein-GSH moieties. As shown in Figure 2, successive elimination of one, two, and three chlorine atoms from the aromatic nucleus of $Cl_4$-1,4-BQ should lead to the formation of monosubstituted adducts ($Cl_3$BQ-Y), three isomers of disubstituted adducts (2,3-, 2,5-, and 2,6-$Cl_2$BQ-$Y_2$), and trisubstituted adducts (ClBQ-$Y_3$). (Here, in general, Y refers to the protein. However, in disubstituted and trisubstituted adducts, where Y > 1, it could either be a protein or a GSH molecule.)

Tetrachloro-1,2-benzosemiquinone ($Cl_4$-1,2-SQ) and $Cl_4$-1,4-SQ are also electrophilic species and hence should also react with macromolecules to form adducts. Indeed, DNA adducts of benzosemiquinone, a reactive metabolite of benzene, have been characterized in mice treated with benzene (*16*). The free radical of $Cl_4$-1,4-SQ can be delocalized within the aromatic nucleus at ortho and para positions to the original oxygen free radical, making these

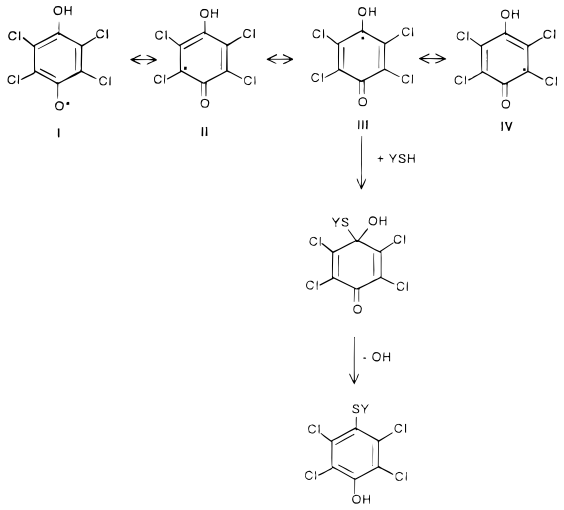


**Figure 3.** Resonance stuctures of $Cl_4$-1,4-SQ leading to the formation of cysteinyl adducts.

positions susceptible for reaction with sulfhydryl groups (Figure 3). However, the residence time of the free radical on the carbon with the OH substituent (structure III) is likely to be longer than those involving carbons with Cl substituents (structures II and IV), due to the higher electronegativity of Cl relative to OH. This leads logically to preferential reaction of cysteinyl residues with structure III leading to the formation of *S*-(4-hydroxytetrachlorophenyl) cysteinyl adducts (Figure 3). Similarly, the reaction of $Cl_4$-1,2-SQ with sulfhydryl groups should give rise to *S*-(2-hydroxytetrachlorophenyl) cysteinyl adducts with the removal of an OH group.

The purpose of this study was to characterize PCP-derived protein adducts. An assay was developed to simultaneously quantitate adducts of quinones and semiquinones, based upon the method of Waidyanatha et al. (*14*) which employed Raney nickel to selectively cleave the cysteinyl adducts (*17*). The catalytic cleavage of monosubstituted, disubstituted, and trisubstituted protein adducts of $Cl_4$-1,4-BQ liberated the corresponding quinones, *i.e.*, trichlorobenzoquinone ($Cl_3$BQ), three isomers of dichlorobenzoquinone ($Cl_2$BQ) (2,3-, 2,5-, and 2,6-), and monochlorobenzoquinone (ClBQ), respectively, which were subsequently reduced to the corresponding quinols by ascorbic acid. The treatment of semiquinone-derived adducts with Raney nickel liberated tetrachlorophenols (TCPs). These quinols and TCPs were derivatized by heptafluorobutyrylimidazole (HFBI) prior to analysis by GC-MS. The formation and stability of these adducts with Hb and Alb were investigated in Sprague-Dawley rats by employing a combination of in vitro and in vivo experiments.

## Materials and Methods

**Chemicals.** 2,3,4,5-TCP (99%+) was purchased from Cambridge Isotope Laboratories (Woburn, MA). 2,3,5,6-TCP (99%+), 2,6-$Cl_2$BQ (99%), and dichlororesorcinol (98%) were obtained from TCI America (Portland, OR). 2,5- Dichlorohydroquinone (2,5-$Cl_2$HQ) (99%+) was obtained from Eastman Kodak (Rochester, NY). Antimony pentachloride, hydroquinone (99%+), $Cl_4$-1,4-BQ (99%), PCP (99%), Raney nickel (pore size 50 $\mu$m, 50% slurry in water), and bis[tris(hydroxymethyl)methylamino]propane (bis-tris-propane buffer) were obthained from Aldrich Chemical Co. (Milwaukee, WI). HFBI was purchased from Pierce (Rockford, IL). Protease XIV (Pronase E), monochlorohydroquinone (ClHQ) (90%), and diethylenetriaminepentaacetic acid (DTPA) were obtained from

**Table 1. Adduct Levels of Proteins Modified with [$^{13}C_6$]$Cl_4$-1,4-BQ, in Vitro**[a]

| adduct | Hb (nmol/g) | Alb (nmol/g) |
|---|---|---|
| [$^{13}C_6$]$Cl_3$BQ-Y | 856 ± 43 | 31.9 ± 0.917 |
| [$^{13}C_6$]2,3-$Cl_2$BQ-$Y_2$ | 1650 ± 34 | 4.04 ± 0.084 |
| [$^{13}C_6$]2,5- and 2,6-$Cl_2$BQ-$Y_2$ | 287 ± 10 | 1.76 ± 0.026 |

[a] Sample means and standard errors are shown for 22−25 measurements of each adduct.

Sigma Chemical Co. (St. Louis, MO). Ascorbic acid, acetic acid, hydrochloric acid (conc), acetone (nanograde), hexane, and methylene chloride (pesticide grade) were obthained from Fisher Scientific (Pittsburgh, PA). Methyl-*tert*-butyl ether (MTBE) and diethyl ether were obtained from Mallinckrodt, Inc. (Paris, KY), and anhydrous $Na_2SO_4$ was obthained from J. T. Baker Co. (Marrietta, GA). Trichlorohydroquinone ($Cl_3$HQ) was synthesized as described in Waidyanatha et al. (1994). TLC plates (silica gel 60 A, 100 $\mu$m) were obtained from Whatman Inc. (Clifton, NJ). All reagents were used without further purification. Water was purified with a Milli-Q system (Waters, Millipore Division, Bedford, MA).

**Synthesis of 2,3-$Cl_2$HQ.** 2,3-$Cl_2$HQ was synthesized according to the method of Rettig and Latscha with some modifications (*18*). Briefly, 5 mmol of hydroquinone was dissolved in 4−5 mL of diethyl ether and added to 45 mL of absolute methylene chloride. To this, 10 mmol of antimony pentachloride was added with stirring, and the reaction was continued at room temperature for 30 min. The reaction mixture was washed four times with 25% aqueous acetic acid, and the organic phase was evaporated in vacuo. The crude mixture was recrystallized from acetone and water. GC-MS analysis of the crude mixture in electron ionization (EI) mode showed the presence of all three isomers of $Cl_2$HQ. 2,3-$Cl_2$HQ was purified from the mixture by preparative TLC (silica gel 60 A, 100 $\mu$m) using methylene chloride and hexane (1:4), and the structure was confirmed by GC-EI-MS. Stock solutions were prepared in acetone, diluted in 100 mM aqueous ascorbic acid containing 1 mM DTPA, and stored at −20 °C prior to use.

**Synthesis of 2,6-$Cl_2$HQ.** 2,6-$Cl_2$HQ was prepared by treating 2,6-$Cl_2$BQ with 100 mM ascorbic acid in acetone. The complete conversion of 2,6-$Cl_2$BQ to 2,6-$Cl_2$HQ was confirmed by GC-EI-MS as described below. Standards were prepared from this solution by diluting in 100 mM aqueous ascorbic acid in 1 mM DTPA and were stored at −20 °C prior to use.

**Preparation of Isotopically Labeled Protein-Bound Internal Standards.** [$^{13}C_6$]$Cl_4$-1,4-BQ and isotopically labeled Hb and Alb-bound internal standards were prepared and purified as described by Waidyanatha et al. (*14*). Stock solutions were prepared and stored at −80 °C prior to use. Multiple reactions of [$^{13}C_6$]$Cl_4$-1,4-BQ with sulfhydryl groups of proteins and/or nonprotein thiols form adducts of the general form [$^{13}C_6$]$Cl_x$BQ-$Y_{(4-x)}$, where Y denotes the protein and $1 \leq x \leq 3$, following removal of one or more chlorine atoms from the aromatic nucleus. Reaction of [$^{13}C_6$]$Cl_x$BQ-$Y_{(4-x)}$ with Raney nickel released [$^{13}C_6$]$Cl_x$BQ which was reduced to [$^{13}C_6$]$Cl_x$HQ by ascorbic acid. The modified proteins were digested and assayed for labeled adducts, according to the Raney nickel procedure (see below), using $Cl_3$HQ, 2,3-$Cl_2$HQ, 2,5-$Cl_2$HQ, 2,6-$Cl_2$HQ, and ClHQ as the calibration standards. These protein-bound internal standards were found to contain the adduct levels shown in Table 1. The levels of [$^{13}C_6$]ClBQ-$Y_3$ were low in both Hb and Alb and hence were not determined. Standard curves were prepared, over a working range of 0−30 ng of $Cl_x$HQ, by extracting [$^{13}C_6$]$Cl_x$HQ, $Cl_x$HQ, and dichlororesorcinol from media equivalent to those of the samples.

**Modification of Rat Blood with $Cl_4$-1,4-BQ in Vitro.** Fifty microliters of solutions of $Cl_4$-1,4-BQ in ethanol was added to 2.95-mL portions of fresh blood obtained from Sprague-Dawley rats, to give final concentrations of 0, 1.5, 5, 15, and 45 $\mu$M. Samples were incubated at 37 °C for 3 h and were mixed every 15−30 min by gentle inversion of the tubes.

**Administration of PCP to Rats.** Twelve to fourteen week-old, male, Sprague-Dawley rats (320−375 g) were obtained from Charles River Breeding Laboratories. Animals were housed in polycarbonate cages and were maintained on a 12 h light/dark cycle for at least 2 weeks before use. Food and water were provided ad libitum. PCP (552 mg) was dissolved in 2 mL of 5 M NaOH and 10 mM phosphate-buffered saline (pH 7.4) to make a 4 mg/mL working solution. In the first experiment, five groups of three animals were given a single dosage of PCP via gastric intubation using a dosing volume of 5 mL/kg at 0 (control), 5, 10, 20, and 40 mg/kg body weight. Control animals were given 10 mM phosphate-buffered saline. Animals were sacrificed 24 h following administration of PCP, and blood was collected by cardiac puncture into a heparinized syringe. In the second experiment animals were divided into eight groups of three, and a single oral dosage of PCP (20 mg/kg body weight) was administered by gastric intubation as described above. The animals were anaesthetized with methoxyfluorane 2, 4, 8, 24, 48, 168, and 336 h following administration of PCP, and the blood was collected as described above.

**Isolation of Globin and Alb.** Red blood cells were separated from plasma by centrifuging at 800*g* for 15 min and were washed three times with an equal volume of physiological saline. Red blood cells and plasma were stored at −80 °C prior to further processing. Globin was isolated from red blood cells as described by Waidyanatha et al. (*14*) except that small molecules were removed from the Hb solution by exhaustive dialysis against 1 mM ascorbic acid at 4 °C using Spectra-Por 1 dialysis tubing (MWCO 6000−8000) with three changes of the buffer. Alb was isolated from plasma as described by Waidyanatha et al. (*14*) except that 50% ammonium sulfate saturation was used to precipitate the immunoglobulins.

**Characterization of Adducts.** Alb (180 mg), isolated from rats given 20 mg of PCP per kg body weight and sacrificed after 8 and 24 h, was used to characterize the adducts formed in vivo. Protein was digested with protease, reacted with Raney nickel to release the sulfur-bound adducts, and derivatized with HFBI as described below. Samples were analyzed by GC-MS in NICI (negative ion chemical ionization) mode by scanning the mass spectrometer from 160 to 610 as described below. Two hundred and fifty to five hundred picograms each of ClHQ, 2,3-$Cl_2$HQ, 2,5-$Cl_2$HQ, 2,6-$Cl_2$HQ, $Cl_3$HQ, 2,3,4,5-TCP, and 2,3,5,6-TCP was derivatized with HFBI and analyzed by GC-NICI-MS under the same conditions.

**Measurement of Adducts.** The procedure used to analyze the protein adducts is outlined in Figure 4. To the purified protein (15−50 mg) dissolved in deionized water was added 1 mL of 0.9 M ascorbic acid, 1 mL of 1 M bis-tris-propane buffer (pH 7), and the corresponding isotopically labeled protein-bound internal standard. The pH of the medium was adjusted to 7 with 5 M NaOH, and the total volume was brought to 6 mL with deionized water. Following the digestion of the protein with

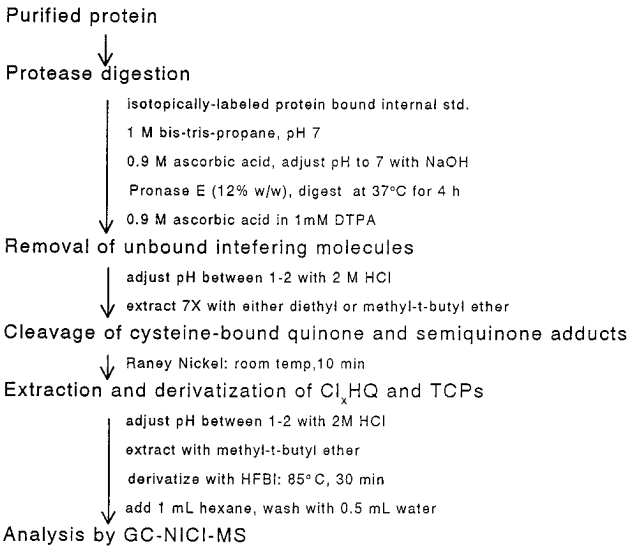


**Figure 4.** Outline of the assay to measure PCP-derived quinone and semiquinone adducts.

protease (12% w/w) at 37 °C for 4 h, 1 mL of 0.9 M ascorbic acid in 1 mM DTPA was added, and the pH of the medium was brought to between 1 and 2 by the addition of 2 M HCl. The aqueous phase was extracted 7× with either diethyl ether or MTBE (10 mL each) to remove unbound TCPs and any other interfering molecules. The last extract was derivatized with HFBI and assayed, as described below, to confirm that unbound TCPs had been completely removed. To the aqueous phase 20 pmol of dichlororesorcinol, an unbound internal standard, was added, and the sample was reacted with Raney nickel (75 mg/mL of reaction volume) at room temperature for 10 min. After the pH of the medium was adjusted to between 1 and 2 with 2 M HCl, the reaction mixture was extracted twice with 8 and 6 mL of MTBE, respectively, and the organic layers were combined and reduced to about 3−4 mL under a stream of nitrogen. After the organic layer was dried with anhydrous $Na_2SO_4$, the extract was reduced to dryness and the vial was sealed under nitrogen. Samples were derivatized with HFBI as described previously (*14*) and analyzed by GC-NICI-MS as described below.

**GC-MS Analysis.** The synthesized products, 2,3-$Cl_2$HQ and 2,6-$Cl_2$HQ, were characterized by GC-EI-MS using a HP 5890 series II gas chromatograph coupled to a HP 5971A mass-selective detector under conditions similar to those given previously (*14*). The column temperature was maintained at 100 °C for 3 min and was then ramped at 10 °C per min to 250 °C, where it was held for 10 min.

The samples from all the other experiments were analyzed by GC-NICI-MS using a HP 5890 gas chromatograph coupled to a HP 5989A MS engine. The column, carrier gas flow rate, injector and MS transferline temperatures, and the chemical ionization reagent gas pressure were the same as reported previously (*14*). The source temperature was maintained at 200 °C. The GC oven temperature was held at 75 °C for 1 min, ramped at 8 °C per min to 160 °C, held for 7 min, and then ramped at 10 °C per min to 200 °C. Late-eluting compounds were removed by ramping the oven temperature at 50 °C per min to 250 °C, where it was held for 10 min. For the characterization of adducts, the instrument was scanned from *m/z* 160 to *m/z* 610. For the quantification of adducts, the following ions were monitored in NICI for the HFB derivatives of TCPs (*m/z* 231),

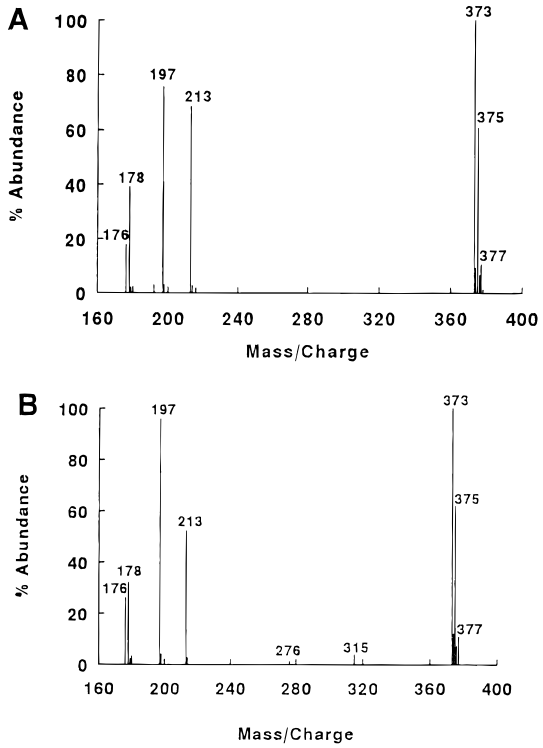


**Figure 5.** NICI spectra of 2,3-$Cl_2$HQ-HFB from (A) an analytical standard and (B) a sample of Alb obtained from a rat to which 20 mg of PCP/kg body weight had been administered.

of ClHQ (*m/z* 339), of 2,3-, 2,5-, and 2,6-$Cl_2$HQ (*m/z* 373), of $Cl_3$HQ (*m/z* 409), of [$^{13}$C]ClHQ (*m/z* 345), of 2,3-, 2,5-, and 2,6-[$^{13}$C]$Cl_2$HQ (*m/z* 379), and of [$^{13}$C]$Cl_3$HQ (*m/z* 415). These ions correspond to loss of a heptafluorobutyryl group from doubly derivatized chlorohydroquinones and singly derivatized TCPs. For chlorohydroquinone derivatives, the quantitation was based on peak areas relative to the corresponding isotopically labeled internal standard. Because the levels of [$^{13}$C]ClHQ were low in both Hb and Alb, quantitation of ClHQ was based on peak areas relative to [$^{13}$C]2,3-$Cl_2$HQ. Since complete chromatographic resolution was not achieved for the HFB derivatives of 2,5- and 2,6-$Cl_2$HQ, both isomers were quantified together. For TCP derivatives, the quantitation was based upon calibration against [$^{13}$C]$Cl_3$HQ. Standard curves for TCP were prepared by extracting [$^{13}$C]$Cl_3$HQ and TCP isomers from media equivalent to those of samples over a working range of 0−30 ng of TCPs.

## Results

**Characterization of Quinone Adducts.** The identities of the quinone adducts, following metabolism of PCP in vivo, were confirmed by assaying Alb from PCP-dosed rats. The GC-NICI-mass spectra of the cleaved adducts matched with those of the corresponding authentic standards. As an example, Figure 5 compares NICI mass spectra of the HFB derivative of 2,3-$Cl_2$HQ arising from metabolism of PCP with that of the corresponding standard. No protein adducts of $Cl_4$-1,2-BQ were detected.

**Characterization of Semiquinone Adducts.** $Cl_4$-1,2-SQ and $Cl_4$-1,4-SQ reacts with cysteinyl residues to form *S*-(2-hydroxytetrachlorophenyl) and *S*-(4-hydroxytetrachlorophenyl) adducts, respectively, which upon cleavage by Raney nickel release 2,3,4,5-TCP and 2,3,5,6-TCP, respectively. The comparison of NICI mass spectra of HFB derivatives of these TCPs with those of authentic standards confirmed the presence of both compounds as illustrated in Figure 6.

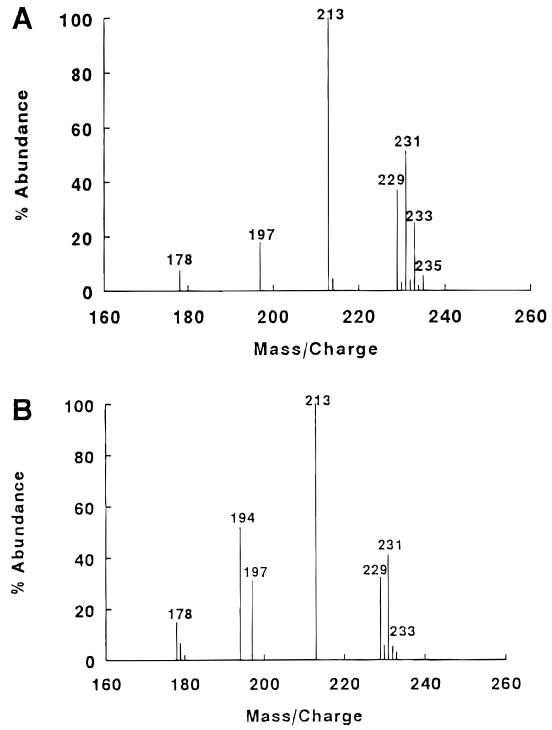


**Figure 6.** NICI spectra of 2,3,4,5-TCP-HFB from (A) an analytical standard and (B) a sample of Alb obtained from a rat to which 20 mg of PCP/kg body weight had been administered.

**Production of $Cl_4$-1,4-BQ Adducts with Hb and Alb in Vitro.** The formation of adducts via multiple reactions of $Cl_4$-1,4-BQ with sulfhydryl groups in proteins was investigated by incubating $Cl_4$-1,4-BQ with rat blood at initial concentrations between 0 and 45 $\mu$M. The levels of monosubstituted, disubstituted, and trisubstituted adducts are presented in Figure 7 panels A and B for Hb and Alb, respectively, versus the initial concentration, $[Cl_4\text{-}1,4\text{-}BQ]_0$, in blood. Linear relationships were observed for all adducts over the entire range of $[Cl_4\text{-}1,4\text{-}BQ]_0$. Results from least-squares regression of adduct concentration on $[Cl_4\text{-}1,4\text{-}BQ]_0$ are shown in Table 2. Note that the slopes are given in nanomolar adducts/micromolar $Cl_4$-1,4-BQ after adjusting adduct levels to a molar basis, assuming that Hb and Alb concentrations were 153 and 16.3 mg/mL of rat blood, respectively (*19*). As observed in previous experiments involving only $Cl_3$BQ-Y (*14*), levels of Alb adducts were much greater than those of Hb for all adducts, with about a 5-fold increase in the slope of Alb adducts relative to those of the corresponding Hb adducts. Moreover, in both proteins, levels of $Cl_3$-BQ-Y were much greater than those of the multisubstituted adducts. The steady decrease in levels of adducts containing 1-, 2-, and 3-substituted sulfhydryl groups suggests possible steric hindrance. Of the disubstituted adducts, levels of the 2,3-$Cl_2$BQ-$Y_2$ adducts were greater than the combined levels of 2,5- and 2,6-$Cl_2$BQ-$Y_2$, suggesting that a second nucleophilic attack is preferred at an ortho position relative to the first nucleophilic attack.

Formation of semiquinone adducts was not observed when blood was modified in vitro with $Cl_4$-1,4-BQ. This lends credence to the notion that $Cl_4$-1,4-SQ-Y and $Cl_4$-1,2-SQ-Y adducts are produced only by semiquinone radicals, since no such radicals could be formed under the in vitro conditions used in these experiments.

**Production of Adducts of Hb and Alb in Vivo.** The formation of PCP-derived adducts of Hb and Alb was investigated in rats over the range of 0 - 40 mg of PCP/kg body weight. Figure 8 panels A and B depict the adduct levels for Hb and Alb, respectively, versus the dosage of PCP. (Note that ClBQ-$Y_3$ adducts are not included in the figure because the levels were below the limit of detection for Hb and were detected in only the three highest dosage groups in Alb.)

Results of least-squares regressions of adduct concentration on the dosage of PCP are given in Table 3. As observed in vitro, the levels of monosubstituted adducts were greater than those of multisubstituted adducts. However, the ratios of slopes of these linear relationships were much different in vivo than those observed in vitro (Table 2). No adducts of $Cl_4$-1,2-BQ were detected in vivo. Regarding the semiquinone adducts, Hb adducts were generally present at higher levels than those of Alb, in contrast to levels of the quinone adducts.

**Stability of Adducts in Vivo.** The stabilties of PCP-derived protein adducts were investigated in Sprague-Dawley rats up to 336 h following administration as illustrated in Figure 9 for $Cl_3$BQ-Y and 2,3-$Cl_2$BQ-$Y_2$. Adduct levels reached maximum values between 8 and 24 h following administration and then declined at rates which were comparable among the different adducts of a given protein.

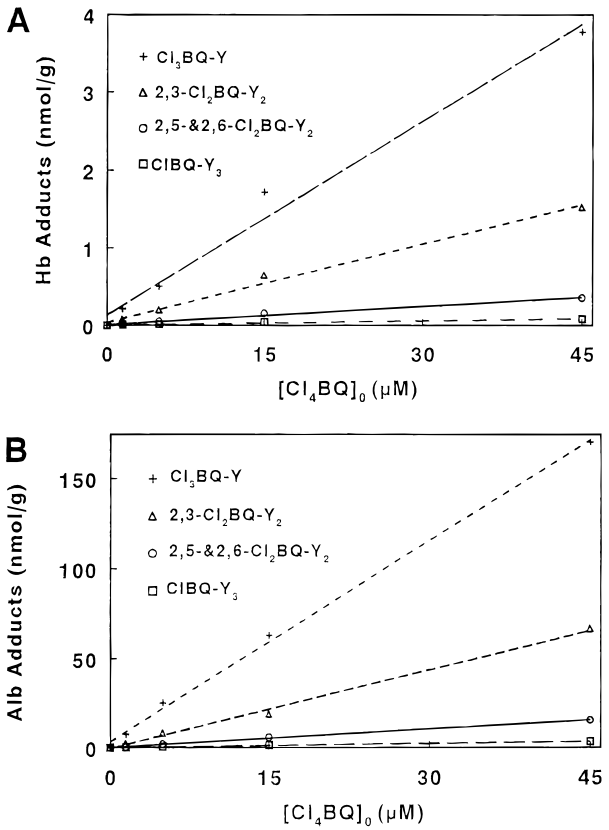


**Figure 7.** Formation of multisubstituted protein adducts of $Cl_4$-1,4-BQ in (A) Hb and (B) Alb, following incubation of Sprague-Dawley rat blood with $Cl_4$-1,4-BQ in vitro.

**Table 2. Linear Regression of Adduct Concentration on $[Cl_4\text{-}1,4\text{-}BQ]_0$ for Blood Reacted with $Cl_4$-1,4-BQ in Vitro[a]**

| | slope, nM adduct/$\mu$M $Cl_4$-1,4-BQ | |
|---|---|---|
| adduct | Hb | Alb |
| $Cl_3$BQ-Y | 12.7 | 61.1 |
| 2,3-$Cl_2$BQ-$Y_2$ | 5.20 | 24.1 |
| 2,5- and 2,6-$Cl_2$BQ-$Y_2$ | 1.07 | 5.66 |
| ClBQ-$Y_3$ | 0.275 | 1.28 |

[a] Data shown in Figure 7.

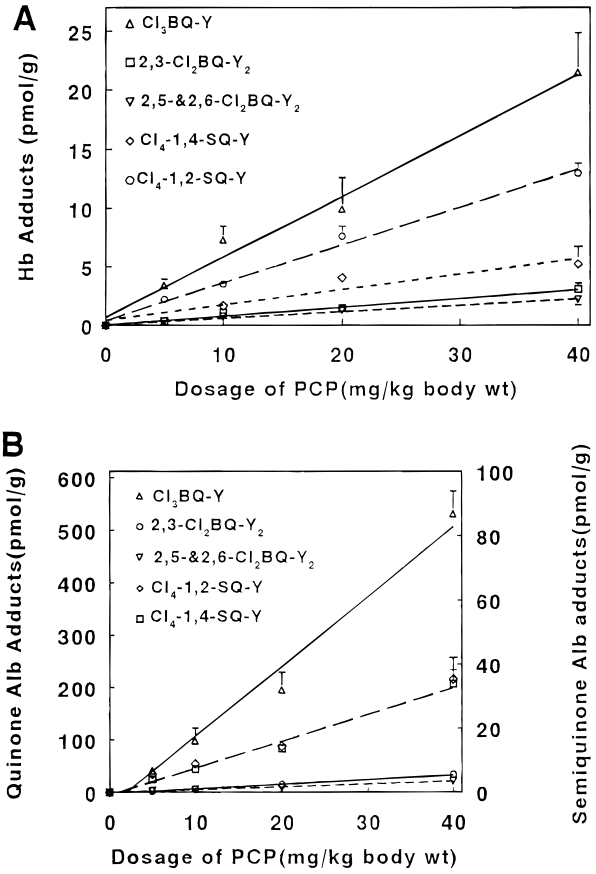


**Figure 8.** Formation of PCP-derived protein adducts in (A) Hb and (B) Alb following administration of PCP to Sprauge-Dawley rats. (Note the different scales and adducts of the two ordinate axes for Alb.) (Mean values and standard errors are shown for three animals per group.)

**Table 3. Linear Regression of Adduct Concentration on Dosage of PCP in Vivo**[a]

| adduct | slope, pM adduct/(mg of PCP/kg body wt)[b] | |
|---|---|---|
| | Hb | Alb |
| $Cl_3BQ$-Y | 79.0 ± 8.84 | 200 ± 13.3 |
| 2,3-$Cl_2BQ$-$Y_2$ | 11.4 ± 1.3 | 14.2 ± 1.65 |
| 2,5- and 2,6-$Cl_2BQ$-$Y_2$ | 8.28 ± 1.18 | 8.75 ± 0.33 |
| $ClBQ$-$Y_3$[c] | ND | 1.06 ± 0.065 |
| $Cl_4$-1,2-SQ-Y | 47.9 ± 3.44 | 13.9 ± 1.47 |
| $Cl_4$-1,4-SQ-Y | 20.2 ± 4.04 | 13.7 ± 0.98 |

[a] Data shown in Figure 8. [b] Mean values and standard errors are shown for three animals per group. [c] $ClBQ$-$Y_3$ levels were low in Hb and hence could not be detected. In Alb this adduct was detected only at the high doses of PCP.

## Discussion

The work reported here extends our earlier investigation of $Cl_3BQ$-Y adducts of Hb and Alb in Sprague-Dawley rats (*14*). On the basis of the low levels of the Hb adducts detected in vivo, we speculated that only a very small fraction of the PCP dose was bioavailable in the systemic circulation as the reactive quinone ($Cl_4$-1,4-BQ). The presence of such small quantities of Hb adducts of $Cl_4$-1,4-BQ also led us to question whether significant levels of other electrophilic species (e.g., semiquinones) might arise from PCP metabolism and whether adducts of $Cl_4$-1,4-BQ might, in fact, be unstable due to continued reaction(s). Here, we describe an assay to simultaneously measure all of the cysteinyl adducts of reactive quinones and semiquinones from PCP metabolism and the characterization of five such adducts in vivo.

It had been shown, in reactions of $Cl_4$-1,4-BQ with GSH in vitro, that $Cl_4$-1,4-BQ retained its oxidized quinone

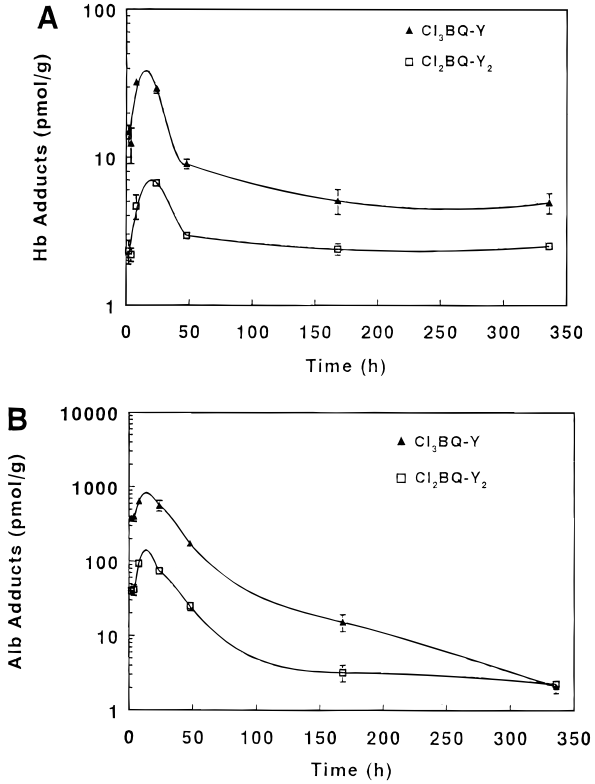


**Figure 9.** Stabilty of PCP-derived protein adducts in Hb and Alb in vivo following administration of 20 mg of PCP/kg body weight to Sprague-Dawley rats. (Note that only two adducts are shown in the figure because the elimination of other adducts was very similar.)

structure after conjugation and continued to react with GSH molecules until all four chlorine atoms on the aromatic ring had been substituted (*15*). We expected $Cl_4$-1,4-BQ to react similarly with protein sulfhydryls to produce multisubstituted adducts, possibly including those with protein−protein cross links. Our results confirmed that $Cl_4$-1,4-BQ did form multisubstituted adducts with both Hb and Alb in rat blood. Given the linear relationships observed in our studies (Figures 7 and 8), we conclude that these multisubstituted adducts were produced at rates that were proportional to the dose of $Cl_4$-1,4-BQ, both in vitro and in vivo (following oral administration of PCP).

Consistent with our preliminary results with adducts of $Cl_4$-1,4-BQ (*14*), the levels of $Cl_3BQ$-Y were higher for Alb than for Hb in vitro (Table 2) and in vivo (Table 3), despite the fact that rat Hb contains a cysteinyl residue ($\beta$-Cys 125) which is typically much more reactive than that of rat Alb (Cys 34) during electrophilic attack in whole blood (*20*,*21*). We speculated previously that this behavior resulted from reduced transport of $Cl_4$-1,4-BQ through cellular membranes, due to either partitioning of this lipophilic species into membrane lipids or covalent binding of the reactive electrophile with membrane proteins. The present study suggests that any such restriction of transport also affects the production of the multisubstituted adducts, particularly in vitro, where the ratios of all forms of Alb adducts were about 5-fold greater than the corresponding Hb adducts (Table 2). However, in going from in vitro to in vivo experiments, the ratio of monosubstituted Alb to Hb adducts dropped from about 5 to about 2.5, and the corresponding ratio for the disubstituted adducts was reduced further to about 1 (Table 3). This difference could be due to a continuous flux of GSH in vivo; however more work is needed to resolve the issue.

Protein adducts of any semiquinone species have not been reported to our knowledge. We have characterized the adducts formed by the reaction of $Cl_4$-1,2-SQ and $Cl_4$-1,4-SQ with protein sulfhydryl groups in vivo. In both Alb and Hb, levels of adducts derived from the quinone species $Cl_4$-1,4-BQ (either as $Cl_3$BQ-Y or as combined multisubstituted adducts) were greater than those derived from the corresponding semiquinone species $Cl_4$-1,4-SQ (Table 3).

Even though no adducts of $Cl_4$-1,2-BQ were detected in the blood of rats to which PCP had been administered, adducts of $Cl_4$-1,2-SQ were detected in both Hb and Alb. In Hb, the levels of $Cl_4$-1,2-SQ-Y were about 2.4-fold higher than those of $Cl_4$-1,4-SQ-Y, whereas in Alb the levels of both types of adducts were similar (Table 3). Regarding preferential reactions of semiquinones between the two proteins, the levels of $Cl_4$-1,2-SQ-Y and $Cl_4$-1,4-SQ-Y were about 3.4- and 1.5-fold higher, respectively, in Hb than in Alb. This is in contrast to what was observed in vivo for the quinone adducts, especially $Cl_3$BQ-Y, where Alb adducts were 2.5-fold higher than the Hb adducts. These observations suggest that either semiquinone species were more readily transported into the erythrocyte than were their quinone analogues or that semiquinone radicals were formed within the erythrocyte per se.

The protein adducts measured in this investigation displayed evidence of biphasic elimination in vivo with a rapid phase ending within 48 h of administration of PCP and a much slower terminal phase, which was more pronounced in Hb than in Alb (see Figure 9). Using the time points between 24 and 336 h following administration of PCP, we estimated that the half-times for elimination of the monosubstituted Hb and Alb adducts were about 155 h and 41 h, respectively. For Hb, this terminal elimination half-time (155 h) was significantly shorter than that anticipated from the normal rate of erythrocyte turnover in the rat, which is 60 days (*22*). Regarding Alb, the estimated half-time (41 h) is slightly shorter than the rate of turnover of serum Alb in the rat, which is reported to be 2−3 days (*23*). In either case, the data suggest that the adducts are somewhat unstable in vivo, probably due to continued reactions with sulfhydryl groups.

In general, the mechanisms most frequently invoked to explain quinone-mediated toxicities involve either sulfhydryl arylation or oxidative stress as a consequence of redox cycling. Recently, the potential importance of quinone-induced protein cross links has also been addressed . For example, the neurotoxicity of 6-hydroxydopamine and 5,6-dihydroxytryptamine is thought to arise, at least in part, from extensive protein cross-linking (*24*). Although our assay cannot differentiate adducts arising from protein−protein cross links from those of proteins and nonprotein thiols, our results are certainly consistent with the notion that protein−protein cross links are formed. Hence, our observation that $Cl_4$-1,4-BQ forms multisubstituted adducts leads us to speculate that protein−protein cross links might play a role in the toxicity of PCP.

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