Investigation of liver binding of pentachlorophenol based upon measurement of protein adducts

Po-Hsiung Lin, Suramya Waidyanatha and Stephen M. Rappaport

Covalent binding of reactive metabolites of pentachlorophenol (PCP) was investigated both *in vitro* and *in vivo* in the livers of male Sprague–Dawley rats via measurement of protein adducts. Cysteinyl adducts of quinones and semiquinones in liver cytosolic (Cp) and nuclear (Np) proteins were assayed after catalytic cleavage by Raney nickel. Results from *in vitro* experiments confirmed that PCP metabolism produced tetrachlorobenzoquinones and the corresponding tetrachlorobenzosemiquinones which subsequently bound to sulphydryl groups in liver proteins. *In vivo*, the production of cysteinyl adducts increased with the administered dosage (0–40 mg PCP per kg body weight) and presented evidence of saturable metabolism. Results suggest two metabolic pathways for PCP, including a high-affinity low-capacity pathway and a low-affinity high-capacity pathway. Time-course experiments *in vivo* and *in vitro* suggested that quinone adducts participated in multiple substitution reactions with protein and/or non-protein thiols, and pointed to possible formation of protein–protein cross-links *in vivo*. The elimination rate constants of quinone adducts *in vitro* were about 0.35 h^{-1} in liver Cp. The elimination of quinone adducts *in vivo* appeared to follow biphasic kinetics with rate constants for the terminal phase being 0.014 and 0.008 h^{-1} in liver Cp and Np, respectively.

Keywords: *protein adducts, liver, pentachlorophenol, benzoquinone, benzosemiquinone.*

Abbreviations: *Alb, albumin; Cl_3CAT, trichlorocatechol; Cl_4CAT, tetrachlorocatechol; ClHQ, monochlorohydroquinone; Cl_2HQ, dichlorohydroquinone; Cl_3HQ, trichlorohydroquinone; Cl_4HQ, tetrachlorohydroquinone; Cl_4-1,2-BQ, tetrachloro-1,2-benzoquinone; Cl_4-1,4-BQ, tetrachloro-1,4-benzoquinone; Cl_3-1,2-BQ-Y, mono-S-substituted protein adduct of tetrachloro-1,2-benzoquinone; Cl_3-1,4-BQ-Y, mono-S-substituted protein adduct of tetrachloro-1,4-benzoquinone; Cl_2-1,4-BQ-Y_2, di-S-substituted protein adduct of tetrachloro-1,4-benzoquinone; Cl-1,4-BQ-Y_3, tri-S-substituted protein adduct of tetrachloro-1,4-benzoquinone; Cl_4-1,2-SQ, tetrachloro-1,2-benzosemiquinone; Cl_4-1,4-SQ, tetrachloro-1,4-benzosemiquinone; 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; Cp, liver cytosolic proteins; EDTA, ethylenediaminetetraacetic acid; EI, electron ionization; Hb, haemoglobin; HFBI, heptafluorobutyrylimidazole; HQ, hydroquinone; MTBE, methyl-tert-butyl ether; NICI, negative ion chemical ionization; Np, acid-soluble nuclear proteins of liver; PCP, pentachlorophenol; PMSF, phenylmethylsulphonylfluoride; PPB, potassium phosphate buffer; TCP, tetrachlorophenol; 1,4-BQ-Y_4, tetra-S-substituted protein adduct of tetrachloro-1,4-benzoquinone.*

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Introduction

The liver has been shown to be the primary site of metabolic transformation of pentachlorophenol (PCP) in rats and mice (Jakobson and Yllner 1971, Renner and Hopfer 1990). Experiments have demonstrated that liver microsomal enzymes (cytochrome P4501A2) convert PCP to the quinols, tetrachlorohydroquinone (Cl_4HQ) and tetrachlorocatechol (Cl_4CAT) (Tashiro *et al.* 1970, Jakobson and Yllner 1971, Ahlborg *et al.* 1974, 1978, van Ommen *et al.* 1986, 1988). It has been speculated that these quinols are oxidized in the liver to the binding species, tetrachloro-1,4-benzoquinone (Cl_4-1,4-BQ) and tetrachloro-1,2-benzoquinone (Cl_4-1,2-BQ), respectively, via the corresponding semiquinones, i.e. tetrachloro-1,4-benzosemiquinone (Cl_4-1,4-SQ) and tetrachloro-1,2-benzosemiquinone (Cl_4-1,2-SQ), which are also capable of binding to macromolecules (van Ommen *et al.* 1986, Waidyanatha *et al.* 1994, 1996). An alternative pathway for direct conversion of PCP to Cl_4-1,4-BQ, but not to Cl_4-1,2-BQ, has been reported to be mediated by peroxidases (Samokyszyn *et al.* 1995). Chlorinated benzosemiquinones (Cl_4-1,4-SQ and Cl_4-1,2-SQ) could also be formed by the one-electron reduction of the corresponding quinones as suggested by Goeptar *et al.* (1995).

Chronic bioassays of PCP in rats and mice have suggested the liver as a target of toxicity. It has been reported that consumption of drinking water, containing 0.3–3 mM PCP, for up to 4 months caused hepatic injury in male Wistar rats (Villena *et al.* 1992). Chronic oral administration of PCP at 30 mg kg^{-1} day^{-1} for 24 months induced liver tumours in MRC–Wistar rats (Mirvish *et al.* 1991) but not in Sprague–Dawley rats (Schwetz *et al.* 1978). However, the Sprague–Dawley rats had elevated levels of serum glutamic pyruvic transaminase. B6C3F1 mice, receiving between 17 and 118 mg PCP kg^{-1} body weight day^{-1} via food for 24 months, had significant increases in liver tumours, with males being more susceptible than females (McConnell *et al.* 1991).

If PCP is, indeed, responsible for toxic effects upon the liver, it is reasonable to expect that the quinones (Cl_4-1,2-BQ and Cl_4-1,4-BQ) and semiquinones (Cl_4-1,2-SQ and Cl_4-1,4-SQ) would play some role. However, no direct evidence for the formation of these reactive species has been reported in the livers of rats to which PCP had been administered. Thus, we undertook, the current investigation to confirm the presence of these reactive species in the liver by measuring their corresponding cysteinyl adducts in liver

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proteins. These adducts serve as biomarkers to reflect the extent of covalent binding of PCP-derived reactive species, namely the quinones and semiquinones. Once the reaction kinetics leading to formation and elimination of these adducts are established, tissue doses of the respective species in the target organs can be estimated.

The assay, which employs Raney nickel to selectively cleave cysteinyl adducts, had previously been applied to measure Cl_4-1,4-BQ adducts in haemoglobin (Hb) and albumin (Alb) in blood (Waidyanatha *et al.* 1994; 1996). We expected the Raney nickel assay to be appropriate for investigating adducts of liver proteins due to the presence of numerous free cysteine residues in liver proteins. For example, the cytosolic proteins (Cp) are known to include glutathione-S-transferases, which contain free cysteines (such as Cys 47 in rat cytosolic glutathione-S-transferase 7-7, Taima *et al.* 1990) and have been shown to produce cysteinyl adducts with Cl_4-1,4-BQ *in vitro* (van Ommen *et al.* 1989). Likewise, the acid-soluble nuclear proteins (Np), of which the histones are the main constituents (Boffa and Vidali 1971, Boffa and Bolognesi 1986), contain free cysteine residues. For example histone H3 contains a free cysteine (Cys 110 in mammals, von Holt *et al.* 1989), as do the non-histone proteins in rat liver nuclei (Boffa *et al.* 1987, Roy and Pathak 1993). Furthermore, cysteine adducts of Np, for various carcinogenic substances, have been characterized both *in vitro* and *in vivo* (Boffa and Bolognesi 1986, Yu *et al.* 1988, Stacks 1990).

In this study we measured the cysteinyl adducts of quinone and semiquinone metabolites of PCP in both Cp and Np fractions of liver proteins in Sprague–Dawley rats. In a parallel investigation of adducts of Hb and serum Alb in the same animals, we observed that the Cl_4-1,4-BQ remained in the oxidized (quinone) form after an initial reaction with a sulphydryl group and continued to react with additional sulphydryl groups thereafter (Waidyanatha *et al.* 1996). This led to refinements in the assay to allow measurement of adducts of chlorinated benzoquinones which were bound to 1–4 cysteinyl residues as well as of protein adducts of the chlorinated semiquinones. In this work, we confirm the hepatic production of multi-S-substituted adducts of Cl_4-1,4-BQ as well as of adducts of Cl_4-1,2-SQ and Cl_4-1,4-SQ following metabolism of PCP *in vivo*. We also observed the production of monosubstituted adducts of Cl_4-1,2-BQ in rat liver nuclei following PCP metabolism *in vitro*.

Structures of the reactive intermediates arising from PCP metabolism, and the corresponding cysteinyl adducts are shown in Figure 1. We designate the products of multiple reactions of Cl_4-1,4-BQ with cysteine residues as Cl_x-1,4-BQ-$Y_{(4-x)}$, where Y denotes a sulphydryl group, and $0 \leq x \leq 3$ represents the number of chlorine atoms remaining on the aromatic nucleus. We designate the monosubstituted cysteinyl adducts of Cl_4-1,2-BQ, Cl_4-1,4-SQ, and Cl_4-1,2-SQ as Cl_3-1,2-BQ-Y, Cl_4-1,4-SQ-Y, and Cl_4-1,2-SQ-Y, respectively.

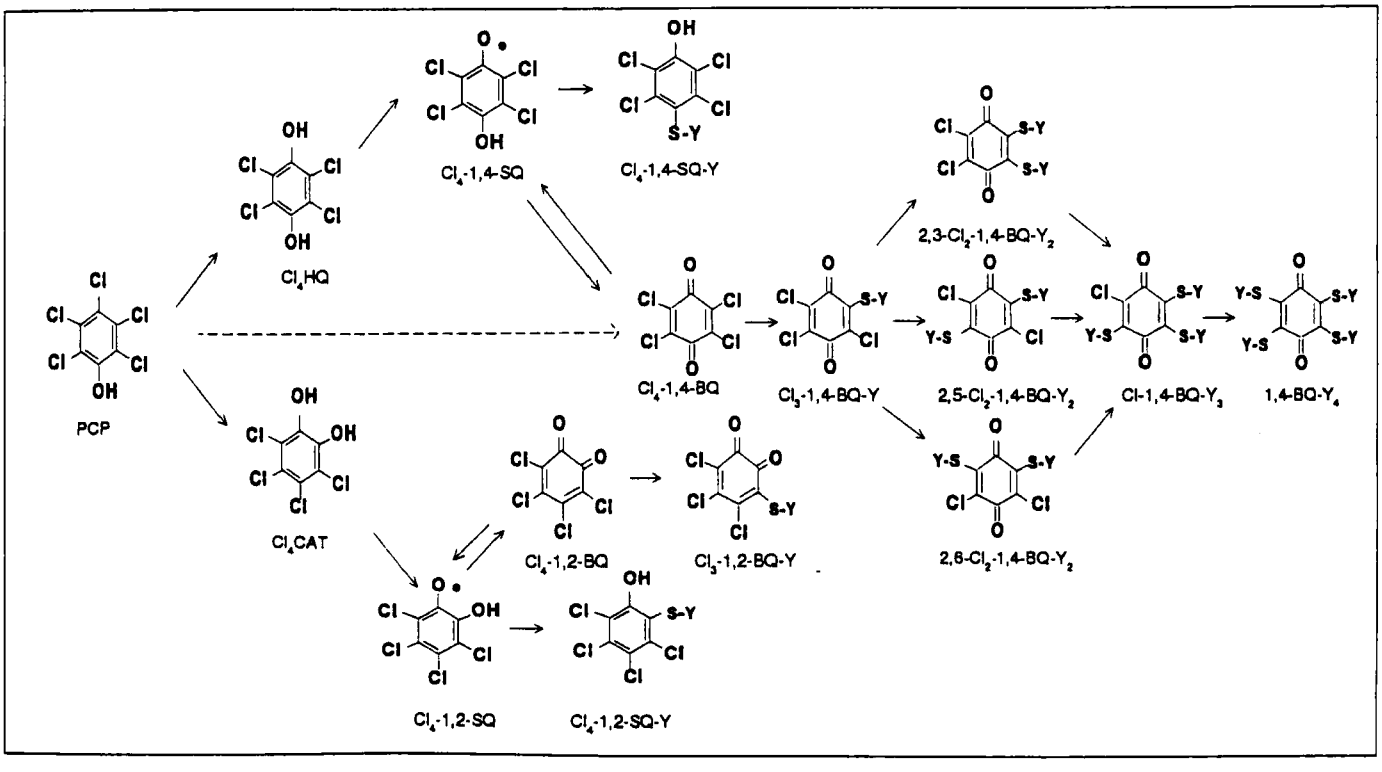

Figure 1. Structures of putative reactive metabolites arising from PCP, and expected cysteinyl adducts of these reactive species.

MATERIALS AND METHODS

Chemicals

PCP (99% pure), Cl_4-1,4-BQ (98% pure), sucrose, phenylmethylsulphonyl fluoride (PMSF), Raney nickel (pore size 50 μm, 50% slurry in water), hydroquinone (HQ), and bis[tris(hydroxymethyl)methylamino]propane (bis-tris-propane buffer) (99%+) were from Aldrich Chemical Company (Milwaukee, WI).

Heptafluorobutyrylimidazole (HFBI) was from Pierce (Rockford, IL). Protease XIV (Pronase E), ethylenediaminetetraacetic acid (EDTA), and monochlorohydroquinone (ClHQ, 90% pure) were from Sigma Chemical Company (St Louis, MO). Ascorbic acid, acetic acid, hydrochloric acid (10 M), hexane (pesticide grade) were from Fisher Scientific (Pittsburgh, PA). Methyl-*tert*-butyl ether (MTBE) was from Mallinckrodt, Inc. (Paris, KY). Anhydrous Na_2SO_4 was from J. T. Baker Company (Marrietta, GA). 2,3,5,6-Tetrachlorophenol (2,3,5,6-TCP) (99%+), 2,6-dichloro-1,4-benzoquinone (99%) and 4,6-dichloroesorcinol (98%) were from TCI America, Inc. (Portland, OR). 2,5-Dichlorohydroquinone (2,5-Cl_2HQ) (99%+) was from Eastman Kodak (Rochester, NY). 2,3,4,5-TCP (99%+), and 3,4,5-trichloro-1,2-benzoquinone were from Cambridge Isotope Laboratories (Woburn, MA). Trichlorohydroquinone (Cl_3HQ), [$^{13}C_6$]Cl_4-1,4-BQ, and 2,3-Cl_2HQ were synthesised as described in Waidyanatha *et al.* (1994, 1996). All chemicals were used without further purification, except for hydrochloric acid, which was purified by extracting with hexane. Water was purified with a Milli-Q system (Waters, Millipore Division, Bedford, MA).

Administration of PCP to animals

PCP was administered to male Sprague–Dawley rats (320–375 g) by gavage as described in Waidyanatha *et al.* (1996). Briefly, two experiments were performed, i.e. one to determine the production of adducts at various dosages of PCP and the other to investigate the stability of the adducts after formation. In the first experiment, 15 animals were assigned to five groups (three animals per group) and each received a single dosage of PCP (0, 5, 10, 20, 40 mg kg^{-1} body weight), prepared in phosphate-buffered saline. Animals were sacrificed 24 h after administration. In the second experiment, eight groups of three animals were given a single dosage of PCP at 20 mg kg^{-1} body weight and were sacrificed at 2, 4, 8, 24, 48, 168, and 336 h after dosing. In both experiments, control rats were given 10 mM phosphate-buffered saline. Immediately after collection of the blood by cardiac puncture, livers were perfused with 0.25 M sucrose solution (10 ml per 100 g body weight), excised, washed in 0.25 M sucrose solution, and frozen at –80 °C until further processing.

Isolation of liver Cp

Approximately 5 g of frozen liver were thawed, sliced, and homogenized in two volumes of 0.25 M sucrose (ice-cold), containing 1 mM EDTA and 1 mM PMSF, with 10–15 strokes using a Potter-Elhjem tissue grinder equipped with a Teflon-coated pestle. The homogenate was filtered through four layers of cheesecloth to remove unbroken cells and was centrifuged at 1000 × *g* for 10 min. The resulting supernatant and pellet were used to isolate liver Cp and Np, respectively. The supernatant was centrifuged at 15 000 × *g* for 20 min to remove the mitochondria, and the resulting supernatant was ultracentrifuged at 105000 × *g* for 60 min to remove the microsomes. The 105 000 × *g*-supernatant was dialysed (Spectra Por 1, dialysis tubing, molecular weight cut-off; 6000–8000) against 3.5 l of 10 mM ascorbic acid solution containing 0.05 mM PMSF with three changes of the buffer. The dialysate was then lyophilized to yield Cp [24.5 mg protein per g of liver (SE = 2.01, *N* = 15)].

Isolation of liver nuclei and Np

The isolation of rat liver nuclei followed the method of Blobel and Potter (1966) with the modifications described in Roy and Pathak (1993). Np was isolated from liver nuclei as described by Boffa and Vidali (1971). In brief, the pellet of the liver homogenate, from centrifugation at 1000 × *g*, was resuspended in one volume of 0.25 M sucrose containing 1 mM EDTA and 0.2 mM PMSF, and was underlaid with four volumes of 2.3 M sucrose, containing 1 mM EDTA and 0.2 mM PMSF. The nuclei were isolated by ultracentrifugation at 105 000 × *g* for 60 min, resuspended in 0.25 M sucrose containing 1 mMEDTA and 0.2 mM PMSF, and centrifuged at 105 000 × *g* for 30 min. The resulting pellet, yielding purified nuclei, was extracted twice with 10 ml of 0.25 M HCl. Extracts were combined, dialysed, and dried as described above, to yield Np [11.1 mg protein per g of liver (SE = 1.01, *N* = 15)].

Reaction of Cl_4-1,4-BQ and Cl_4HQ with liver proteins in vitro

Reaction of Cl_4-1,4-BQ with liver proteins was investigated in both liver homogenates and isolated liver Cp and Np. Liver homogenates were prepared in 0.25 M sucrose (containing 1 mM PMSF and 1 mM EDTA) to give a protein concentration of 7.7 mg ml^{-1} (SE = 0.3, *N* = 12) for Cp and 2.0 mg ml^{-1} (SE = 0.8, *N* = 12) for Np. Isolated Cp and Np were resuspended in 10 mM potassium phosphate buffer (PPB) (pH 7.4) to give a protein concentration of 10 mg ml^{-1}. To these preparations Cl_4-1,4-BQ (dissolved in acetone) was added to give final Cl_4-1,4-BQ concentrations from 0 to 400 μM (liver homogenate) or from 0 to 2 μM (isolated liver proteins). After 15 min of incubation at 37 °C, the reaction was terminated by adding ascorbic acid to a final concentration of 10 mM and placing the mixture in an ice bath.

In a separate experiment, 20 μl of either Cl_4HQ (dissolved in acetone containing 1 mM ascorbic acid), or Cl_4-1,4-BQ (dissolved in acetone) was added to 2 ml liver homogenate to give final Cl_4-1,4-BQ concentrations from 0 to 400 μM. The reaction was carried out at 37 °C for 3 h and was terminated by adding ascorbic acid as noted above.

Reaction of PCP with isolated liver nuclei in vivo

The purity of freshly isolated liver nuclei was checked under a light microscope. The nuclei (5.6 mg of Np per ml) were resuspended in 10 mM PPB (pH 7.4) containing 0.2 mM PMSF and were pre-incubated in a water bath at 37 °C for 5 min. PCP (0–100 μM) and NADPH (1 mM) were added to 2 ml portions of the nuclear suspensions which were then incubated at 37 °C for 30 min. [Note: a stock solution of PCP (32 mg ml^{-1}) was prepared by dissolving 90.5 mg PCP in 2 ml of 5 M sodium hydroxide and diluting in 10 mM of PPB.] Nuclei were pelleted by centrifugation at 1000 × *g* for 10 min and Np were isolated as described above.

Stability of liver protein adducts in vitro

Liver Cp and Np (100 mg) isolated from rats dosed with PCP (20 mg kg^{-1} body weight, sacrificed at 2–4 h after dosing) were resuspended in deionized water to make a final concentration of 10 mg ml^{-1}. The solutions were incubated at 37 °C and 1 ml aliquots were withdrawn at time intervals of 0, 1, 2, 4, 8, 21, 46 and 168 h, and assayed for protein adducts as described below.

In a separate experiment, untreated rat liver was homogenized in two volumes of 10 mM PPB (pH 7.4). Cl_4-1,4-BQ (dissolved in acetone) was added to 5.5 ml of liver homogenate to give a final concentration of 10 μM. The homogenate was incubated at 37 °C, and 0.5 ml aliquots were withdrawn at time intervals of 0.5, 1, 3, 5, 15, 60, 180, and 360 min. Cp was isolated and assayed for protein adducts as described below.

Synthesis of isotopically-labelled protein-bound internal standards

Isotopically-labelled protein-bound internal standards were prepared by reacting the 100000 × *g* supernatant of liver homogenate (containing 120 mg of liver Cp), and calf thymus histones (containing 250 mg of histones) (Fluka Chemical Co.) dissolved in deionized water (total volume: 15 ml and 8 ml, respectively) with

Adducts	Adduct level (SE) (nmol g^{-1})[a]	
	Cp	Histones
Cl_3-1,4-BQ-Y	270 (34)	1658 (172)
2,3-Cl_2-1,4-BQ-Y_2	19 (2)	403 (26)
2,5- & 2,6-Cl_2-1,4-BQ-Y_2	17 (1)	90 (6)
Cl-1,4-BQ-Y_3	40 (5)	139 (13)
1,4-BQ-Y_4	9.2 (0.86)	41.0 (4.7)

Table 1. Adduct levels of proteins modified by [$^{13}C_6$]Cl_4-1,4-BQ *in vitro*.
[a] *N* = 5–16 assays for each adduct.

[$^{13}C_6$]Cl_4-1,4-BQ (48% pure, Waidyanatha *et al.* 1994) at 37 °C for 1 and 6 h, respectively. The final concentration of [$^{13}C_6$]Cl_4-1,4-BQ in calf thymus histones was 4.8 mM. In Cp, [$^{13}C_6$]Cl_4-1,4-BQ was added in two portions to give a final concentration of 0.4 mM. After the reaction, the proteins were dialysed, lyophilized as described above, digested and assayed for labelled adducts. Standard curves were prepared by extracting [$^{13}C_6$]ClHQ, [$^{13}C_6$]2,3-Cl_2HQ, [$^{13}C_6$]2,5-Cl_2HQ, [$^{13}C_6$]2,6-Cl_2HQ, [$^{13}C_6$]Cl_3HQ and their corresponding unlabelled hydroquinones, and 4,6-dichloroesorcinol from solutions equivalent to those of the samples. The adduct levels of these modified proteins are given in Table 1.

Analysis of adducts

All cysteinyl adducts arising from the chlorinated benzoquinones and benzosemiquinones were assayed by the Raney nickel procedure as described for Hb and serum Alb (Waidyanatha *et al.* 1996). Briefly, the isolated proteins (5–20 mg) and the isotopically-labelled protein-bound internal standards were digested with protease. After acidfying the medium to pH 3, with 2 M HCl, samples were extracted to 6–10 times with 10 ml of MTBE (this extraction step was used to remove any unbound contaminants, mainly TCPs, from the medium). Fifty pg of 4,6-dichlororesorcinol (an unbound internal standard) was added to the medium and the samples were reacted with Raney nickel. After adjusting the medium to pH 3 with 2 M HCl, samples were extracted two times with 8 ml of MTBE, derivatized with HFBI, and analysed by GC-NICI-MS. The products of this reaction for a quinone adduct and a semiquinone adduct are shown in Figure 2. For the quantitation of adducts, the following fragment ions were monitored in NICI for the respective HFB-derivatives: of TCPs (*m/z* 231); of HQ (*m/z* 305); of ClHQ (*m/z* 339); of 4,6-dichlororesorcinol, Cl_2HQs (*m/z* 373); of Cl_3HQ and Cl_3CAT (*m/z* 409); of [$^{13}C_6$]HQ (*m/z* 311); of [$^{13}C_6$]ClHQ (*m/z* 345); of [$^{13}C_6$]Cl_2HQs (*m/z* 379), and of [$^{13}C_6$]Cl_3HQ (*m/z* 415). These ions correspond to loss of a heptafluorobutyryl group from singly-derivatized TCPs and doubly-derivatized chlorohydroquinones. For the characterization of the product after Raney nickel cleavage of [$^{13}C_6$]1,4-BQ-Y_4, the instrument was scanned from *m/z* 50 to *m/z* 700 in both EI and NICI modes. For ClHQ, Cl_2HQ, and Cl_3HQ, the quantitation was based upon peak areas relative to the corresponding isotopically-labelled internal standard. For TCPs and 3,4,5-Cl_3CAT, the quantitation was based upon calibration against [$^{13}C_6$]Cl_3HQ. For [$^{13}C_6$]HQ, quantitation was based upon calibration against HQ. In all cases, the limit of detection for both quinone and semiquinone adducts was estimated to be 1 pmol g^{-1} when 20 mg of protein was used in the assay. Standard curves were prepared over a range of 0 to 10 ng by extracting authentic standards from solutions equivalent to those of samples, except in the case of HQ, where no protease was added to the solution.

Results

Production of liver protein adducts in vitro

Formation of [$^{13}C_6$]1,4-BQ-Y_4

In a parallel investigation, we showed that cysteinyl adducts of Cl_4-1,4-BQ in blood proteins produced multi-S-substituted adducts via additional reactions with proteins and/or non-protein thiols (Waidyanatha *et al.* 1996). Although, we proved the presence of three di-S-substituted and one tri-S-substituted adducts in that study, we could not confirm the presence of the tetra-S-substituted adducts (1,4-BQ-Y_4), due to the presence of high background levels arising from 1,4-benzoquinone (McDonald *et al.* 1993). In the present study, we measured [$^{13}C_6$]1,4-BQ-Y_4 in the [$^{13}C_6$]Cl_4-1,4-BQ-modified proteins which

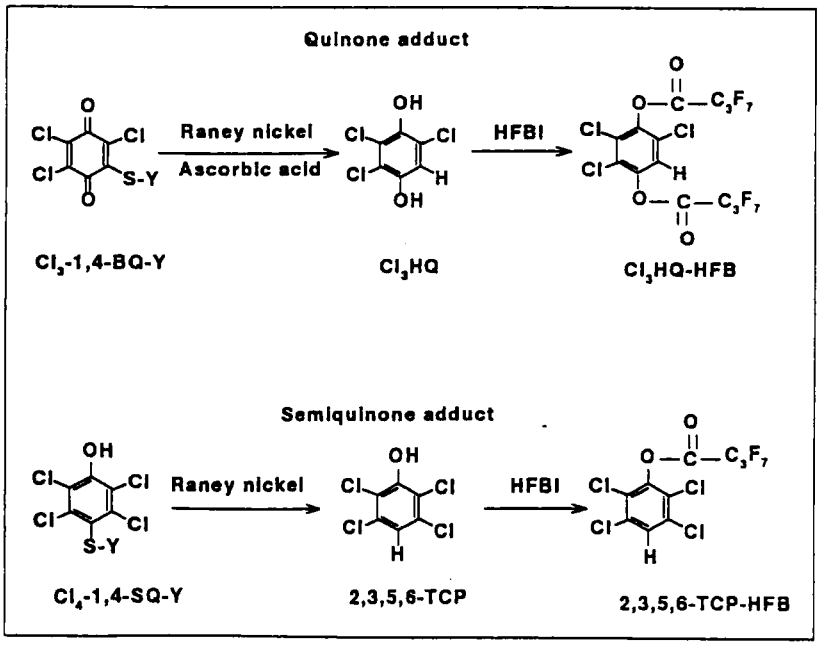

Figure 2. The reaction of a quinone adduct (above) and a semiquinone adduct (below) with Raney nickel followed by subsequent derivatization with HFBI.

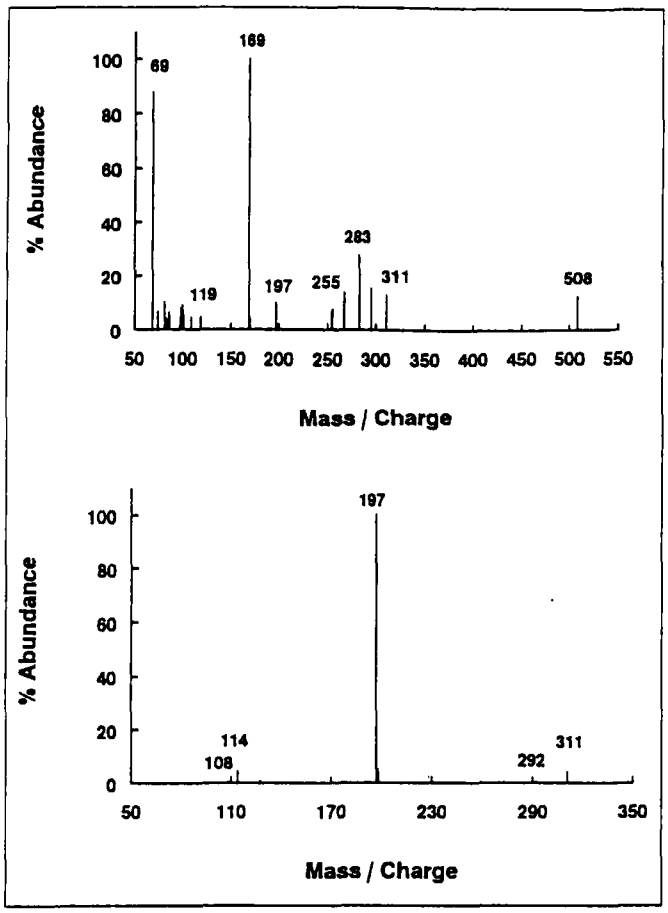

Figure 3. EI mass spectrum (above) and NICI mass spectrum (below) of [$^{13}C_6$]HQ-HFB from histone modified *in vitro* with [$^{13}C_6$]Cl_4-1,4-BQ.

had been prepared for use as internal standards, since these isotopically-labelled adducts could be detected free of background effects. Figure 3 shows the EI- and NICI-mass spectra of [$^{13}C_6$]HQ-HFB derived from [$^{13}C_6$]Cl_4-1,4-BQ-modified calf-thymus histone proteins. The levels of [$^{13}C_6$]1,4-BQ-Y_4 in rat liver Cp and calf-thymus histones were 9.2 (SE = 0.86, N = 5) and 41.0 (SE = 4.7, N = 5) nmol g^{-1} protein, respectively, representing 2.6% and 1.8% of the total cysteinyl adducts in these modified proteins. The ratios of the level of [$^{13}C_6$]Cl_3-1,4-BQ-Y_4 to [$^{13}C_6$]1,4-BQ-Y_4 were about 30 and 40, respectively, for rat liver Cp and calf-thymus histones.

Production of liver protein adducts by Cl_4-1,4-BQ and Cl_4HQ

The formation of cysteinyl adducts was investigated by incubating Cl_4-1,4-BQ with liver homogenates and with isolated liver Cp and Np. Production of multi-S-substituted adducts was observed in all cases. Evidence of non-linear (less than proportional with [Cl_4-1,4-BQ]) production of adducts was observed with Cp but not with Np for all adducts. Results are summarized in Table 2 in terms of the slopes and SE values for least-squares regressions of adduct level on [Cl_4-1,4-BQ] over the indicated linear ranges. Coefficients of determination were large ($0.876 \le r^2 \le 0.993$) in all cases. The slopes shown in Table 2 indicate that the levels of adducts decreased in the order monosubstituted > disubstituted > trisubstituted, for incubation with liver homogenate, and monosubstituted > trisubstituted > disubstituted, for incubation with isolated proteins. The slopes also indicate that production of adducts was greater (1.8–9.3-fold) in Cp than in Np for incubations of liver homogenates, while in isolated proteins, adduct production was comparable in Cp and Np. These data show that, for both Cp and Np, adduct levels were generally greater in isolated proteins than in liver homogenates. No Cl_4-1,4-SQ adducts were detected in these incubations.

In a separate experiment, liver homogenate was incubated for 3 h with either Cl_4-1,4-BQ or Cl_4HQ and adducts were measured in the Cp fraction. The results, summarized in Table 3, show that adducts were produced by incubation of homogenate with Cl_4HQ, confirming that Cl_4HQ was oxidized to Cl_4-1,4-BQ *in vitro*. Furthermore, the slopes of the regressions were comparable for incubations with Cl_4-1-BQ and Cl_4HQ. Finally, comparison of the slopes for Cp from the incubation with Cl_4-1,4-BQ in liver homogenate in Tables 2 and 3 indicates that much lower adduct levels were present following incubation for 3 h than for 15 min. This suggests that the chlorinated adducts were rapidly undergoing additional substitution reaction over the course of these experiments.

Incubate	Adducts	Cp		Np	
		Slope (SE) (nmol g^{-1})/(μM)	Linear range[b]	Slope (SE) (nmol g^{-1})/(μM)	Linear range[b]
Homogenate	Cl_3-I,4-BQ-Y	1.99 (0.13)	0–200	1.09 (0.05)	0–400
	2,3-Cl_2-I,4-BQ-Y_2	1.40 (0.05)	0–200	0.348 (0.012)	0–400
	2,5- & 2,6-Cl_2-I,4-BQ-Y_2	0.196 (0.011)	0–200	0.057 (0.002)	0–400
	Cl-I,4-BQ-Y_2	1.30 (0.06)	0–200	0.142 (0.016)	0–400
Isolated proteins	Cl_3-I,4-BQ-Y	8.61 (0.41)	0–2	6.50 (0.64)	0–2
	2,3-Cl_2-I,4-BQ-Y_2	0.984 (0.040)	0–2	1.53 (0.71)	0–2
	2,5- & 2,6-Cl_2-I,4-BQ-Y_2	0.142 (0.006)	0–2	0.458 (0.056)	0–2
	Cl-I,4-BQ-Y_3	3.52 (0.13)	0–2	6.87 (0.39)	0–2

Table 2. Slopes from linear regressions of adduct levels on [Cl_4-1,4-BQ] *in vitro*[a].

[a] Incubation for 15 min.

[b] Linear range of [Cl_4-1,4-BQ] in μM for production of adducts.

Adducts	Cl_4-1,4-BQ		Cl_4HQ	
	Slope (SE) (nmol g^{-1})/(μM)	Linear range[b]	Slope (SE) (nmol g^{-1})/(μM)	Linear range[b]
Cl_3-1,4-BQ-Y	1.22 (0.02)	0–80	0.800 (0.06)	0–80
2,3-Cl_2-1,4-BQ-Y_2	0.166 (0.007)	0–80	0.067 (0.004)	0–80
2,5- & 2,6-Cl_2-1,4-BQ-Y_2	0.050 (0.007)	0–20	0.015 (0.001)	0–80
Cl-1,4-BQ-Y_3	0.245 (0.005)	0–400	0.070 (0.001)	0–80

Table 3. Slopes from linear regressions of Cp adduct levels on [Cl_4-1,4-BQ] and [Cl_4HQ] in liver homogenate[a].
[a] Incubation for 3 h.
[b] Linear range of [Cl_4-1,4-BQ] and [Cl_4HQ] in μM for production of adducts.

Production of liver protein adducts by PCP in isolated rat liver nuclei

The formation of quinone and semiquinone adducts in liver nuclei was investigated by incubation of isolated nuclei with PCP. The conversion of PCP to its reactive metabolites in these incubates was confirmed by the measurement of protein adducts of Cl_4-1,4-BQ, Cl_4-1,2-BQ, Cl_4-1,4-SQ and Cl_4-1,2-SQ in the Np fraction. This is illustrated in Figure 4 for adducts of Cl_4-1,4-BQ and Cl_4-1,2-BQ, and the corresponding semiquinones. As shown in Figure 4, a linear relationship was found for Cl_3-1,4-BQ-Y but non-linear functions were observed for the other species (Cl_3-1,2-BQ-Y, Cl_4-1,2-SQ-Y and Cl_4-1,4-SQ-Y) at [PCP] > 50 μM. The applicability of linear versus non-linear regression to the experimental data was confirmed by residual analysis. Results of least squares regressions of adduct levels on [PCP] are given in Table 4 for the linear ranges of PCP.

Note that we also observed the formation of 3,4,5-Cl_3-1,2-BQ-Y (arising from Cl_4-1,2-BQ) in these incubations. Following the Raney nickel assay, the expected product was identified by comparing the retention time and the mass spectrum (NICI) with those of the authentic standard, 3,4,5-Cl_3CAT (mass spectra not shown). However, we did not detect the other isometric form of the adduct arising from Cl_4-1,2-BQ, i.e. 3,4,6-Cl_3-1,2-BQ-Y.

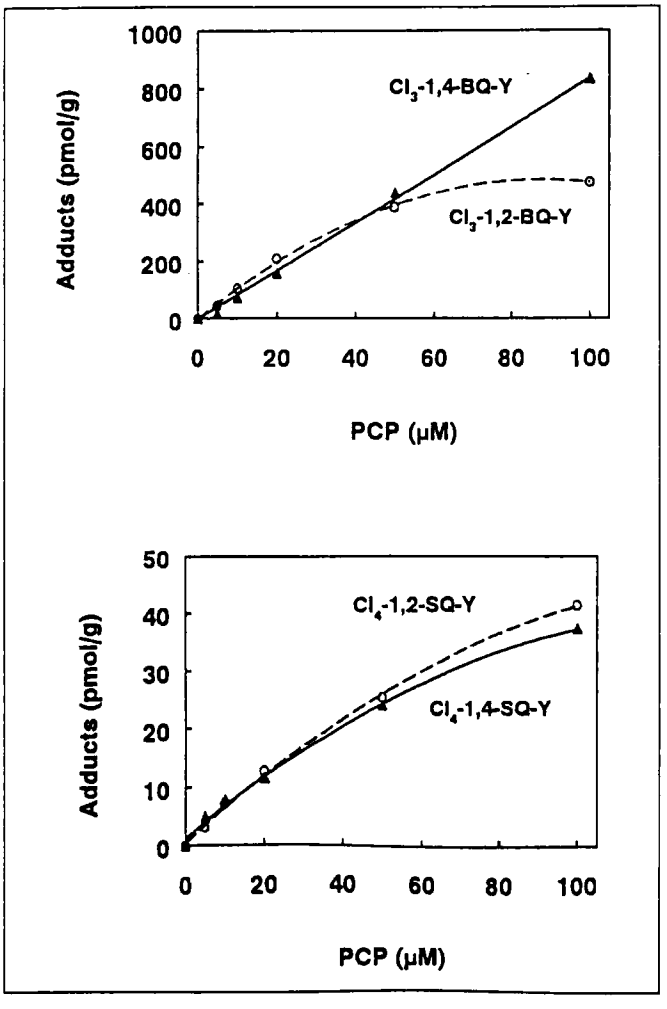

Figure 4. Concentration-dependent production of selected protein adducts by PCP *in vitro* in isolated liver nuclei.

Stability of liver protein adducts *in vitro*

The stability of PCP-derived adducts of liver proteins was investigated by incubating isolated Cp and Np from animals to which 20 mg PCP per kg body weight had been administered 2–4 h prior to sacrifice. Although, in general, the levels of quinone- and semiquinone-derived adducts of both liver Cp and Np were stable over the course of the experiment, slightly increasing levels of Cl_3-1,4-BQ-Y, Cl_2-1,4-BQ-Y_2, and Cl_4-1,4-SQ-Y were observed in Cp. This suggests that some Cl_4HQ was present in these proteins, which is not surprising given that only 2–4 h had elapsed following administration of PCP. The results of this experiment suggest that the PCP-derived adducts of isolated liver proteins are stable *in vitro*, in the absence of small nucleophilic species, such as glutathione.

When untreated liver homogenate was incubated with Cl_4-1,4-BQ (10 μM), the various adducts were all observed to degrade at approximately the same rate after reaching a maximum value at 1 h (Figure 5).

Production of liver protein adducts in vivo

Dose-dependent production of liver protein adducts *in vivo*

Figure 6 depicts the adduct levels measured in liver proteins versus PCP dosage to Sprague–Dawley rats. Approximately linear relationships were observed over the range of dosages between 0 and 20 mg PCP per kg body weight for all Cl_4-1,4-BQ-

Adducts	Slope (SE) (pmol g^{-1})/(μM)	Linear range[b]
Cl_3-1,4-BQ-Y	8.39 (0.14)	0–100
2,3-Cl_2-1,4-BQ-Y_2	5.47 (0.18)	0–50
2,5-& 2,6-Cl_2-1,4-BQ-Y_2	8.63 (0.09)	0–50
Cl-1,4-BQ-Y_3	14.8 (0.7)	0–50
Cl_4-1,2-SQ-Y	0.542 (0.027)	0–50
Cl_4-1,4-SQ-Y	0.515 (0.036)	0–50
Cl_3-1,2-BQ-Y	8.26 (0.50)	0–50

Table 4. Slopes from linear regressions of Np adduct level on [PCP] *in vitro* in isolated liver nuclei[a].

[a] Incubation for 30 min.

[b] Linear range of [PCP] in μM for production of adducts.

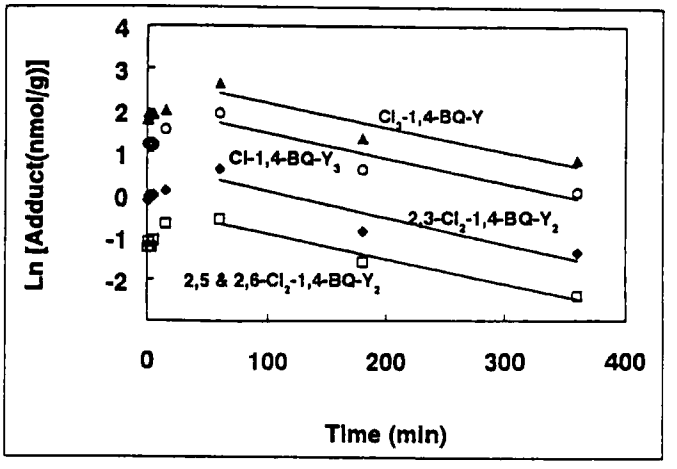

Figure 5. Loss of PCP-derived protein adducts over time in liver Cp *in vitro*. (Mean values are shown for two measurements. Lines represent least-squares regressions for the last three time points.)

derived adducts of Np. However, at 40 mg PCP per kg body weight, the relationships between adduct levels of Cp and Np and dosage became non-linear, with greater than proportional production of adducts in this range. This non-linear relationship between Cp and Np adducts and dosage was confirmed by residual analysis under the linear model. As observed *in vitro*,

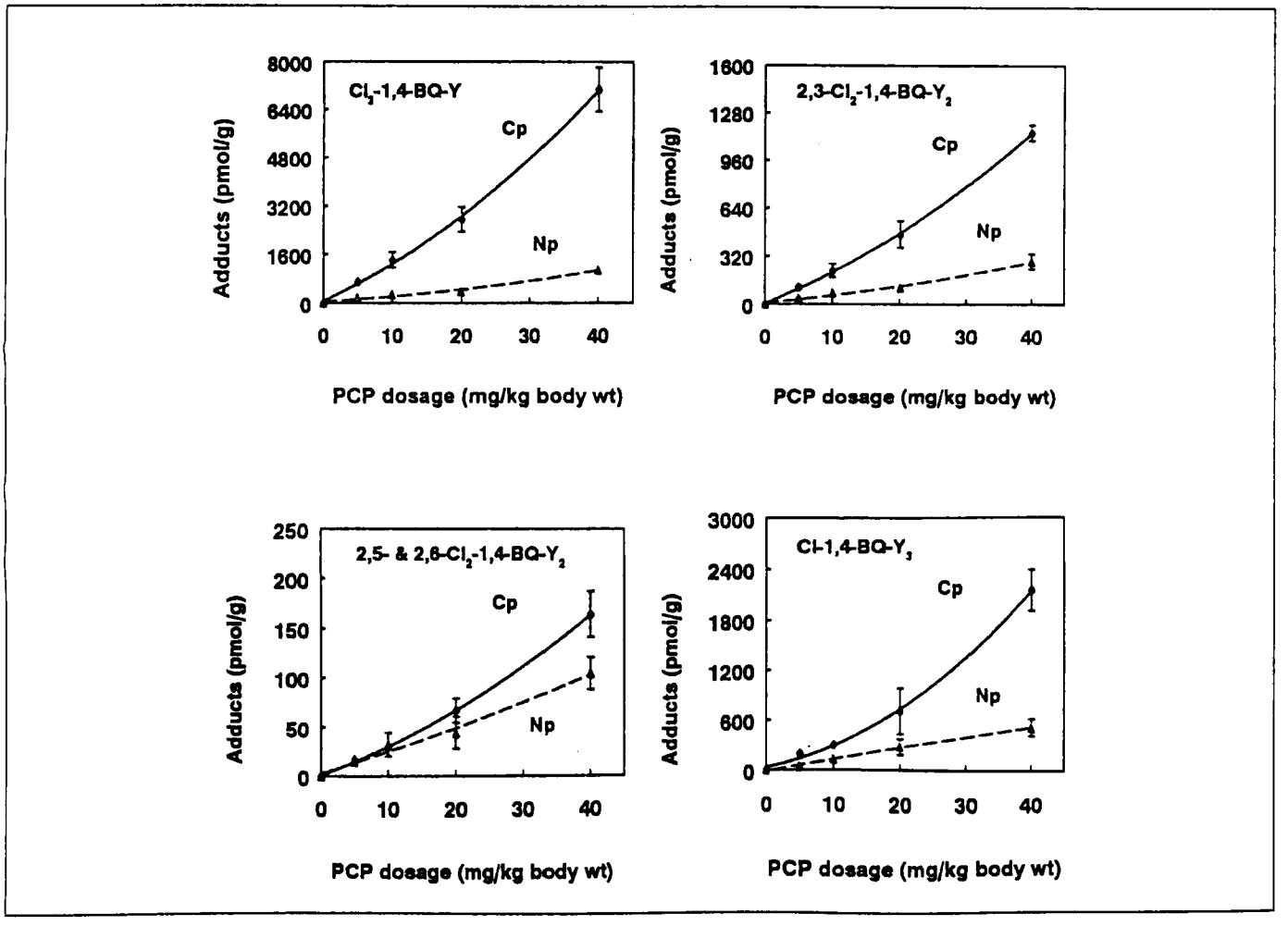

Figure 6. Dose-dependent production of quinone adducts in liver Cp and Np following administration of PCP to rats. (Mean values and standard deviations are shown for three animals per group.)

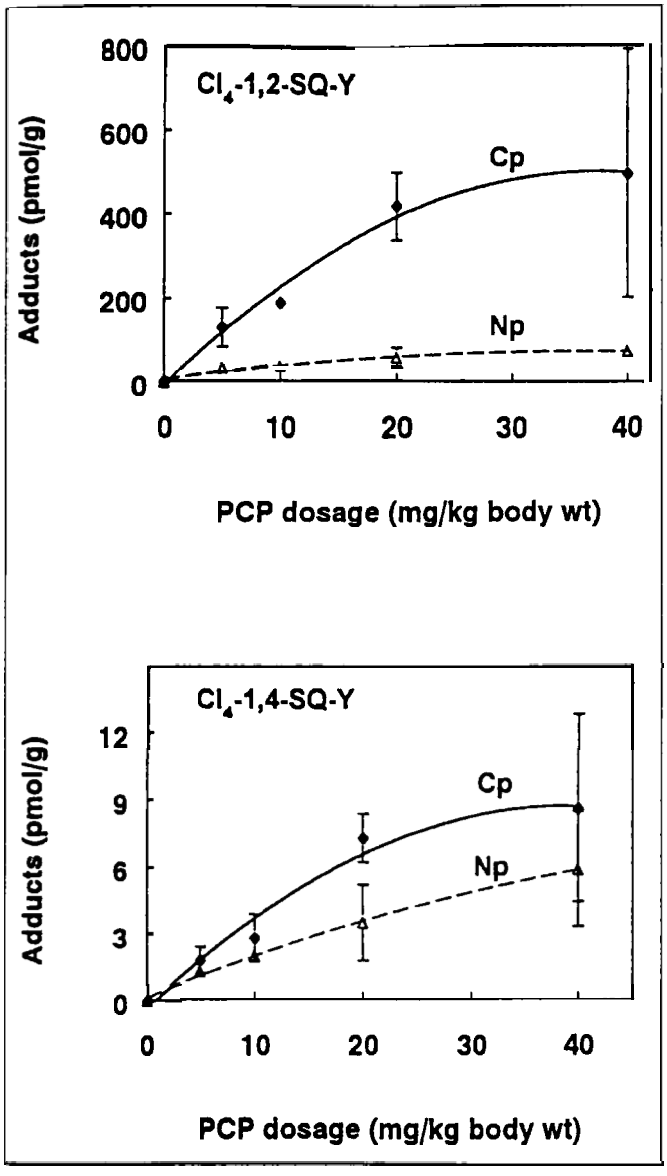

Figure 7. Dose-dependent production of semiquinone adducts in liver Cp and N following administration of PCP to rats. (Mean values and standard deviations ar shown for three animals per group.)

the levels of adducts decreased in the order monosubstituted > disubstituted > trisubstituted and there was greater production of Cp adducts than of Np adducts (about 3–6-fold).

Adducts of both Cl_4-1,2-SQ and Cl_4-1,4-SQ were also detected in liver proteins in the animals dosed with PCP. As shown in Figure 7, the production of Cl_4-1,2-SQ-Y and Cl_4-1,4-SQ-Y increased with dosage of PCP, although levels were less than proportional in Cp at dosages above 20 mg kg^{-1} body weight. The production of adducts of Cl_4-1,2-SQ was much greater than that of Cl_4-1,4-SQ (60-fold for Cp and 15-fold for Np), and for each isomer of the semiquinones, production of Cp adducts was greater than for Np adducts (7-fold greater for Cl_4-1,2-SQ and 2-fold greater for Cl_4-1,4-SQ).

Stability of liver protein adducts *in vivo*

The stability of PCP-derived adducts was investigated in Sprague–Dawley rats up to 336 h following administration of 20 mg PCP per kg body weight. Figures 8 and 9 depict the levels of quinone and semiquinone adduct levels, respectively, versus time post-administration. The maximum levels of all adducts were observed between 2 and 24 h after administration of PCP. In general, semiquinone adducts reached peak concentrations earlier than those of the quinones. After achieving a maximum value, Cl_4-1,4-BQ derived adducts declined over the course of the experiment at rates which were comparable among the different adducts of a given protein. In liver Cp, both Cl_4-1,4-SQ and Cl_4-1,2-SQ adduct levels declined at rates relatively faster than those of the corresponding quinone adducts. In all cases, some evidence of biphasic elimination was apparent with a rapid phase ending between 24
and 48 h and a slower phase continuing thereafter. This behaviour was particularly apparent for the Cl_4-1,4-SQ adducts of Np (Figure 9). The solid lines shown in Figures 8 and 9 represent least squares regressions for the slower phase of elimination, based upon the last three points in each case.

Discussion

The major objective of this work was to use the methodology, developed for measuring adducts of PCP metabolites in blood proteins (Waidyanatha *et al.* 1994, 1996), to investigate protein binding in liver cytosol (Cp) and nuclei (Np). Our results indicate that the primary reactive metabolites of PCP, Cl_4-1,4-BQ, reacts with crude liver homogenate and with isolated liver proteins *in vitro* to produce Cl_3-1,4-BQ-Y, in both Cp and Np, and that Cl_3-1,4-BQ-Y undergoes additional substitution reactions to produce Cl_2-1,4-BQ-Y_2 and Cl-1,4-BQ-Y_3. Since we confirmed the presence of [$^{13}C_6$]-1,4-BQ-Y_4 in proteins modified with [$^{13}C_6$]Cl_4-1,4-BQ *in vitro*, we also infer that Cl-1,4-BQ-Y_3 reacted further to produce 1,4-BQ-Y_4.

The origin of the sulphydryl groups giving rise to the multi-S-substituted adducts is uncertain. The fact that we were able to measure large quantities of Cl_2-1,4-BQ-Y_2 and Cl-1,4-BQ-Y_3, after only 0.5 min of incubation with liver homogenate (Figure 5), suggests that these multiple substitution reactions proceeded rapidly in the presence of free sulphydryl groups (associated, for example, with glutathione) which abound in this medium. However, we also detected multisubstituted adducts following incubation of Cl_4-1,4-BQ with isolated proteins that did not contain small molecules (which had been removed by dialysis); thus, we infer that multiple substitution reactions also proceeded via other proteins, albeit at slower rates, and that such reactions could include protein–protein cross-links. To gauge the relative importance of reactions with small molecules versus other proteins, the ratios of the levels of Cl_3BQ-Y to the sum of Cl_2-1,4-BQ-Y_2 and Cl-1,4-BQ-Y_3 were compared for the experiments involving liver homogenate and isolated proteins. In the homogenates, incubated with Cl_4-1,4-BQ for 15 min (Table 2), this ratio was about 0.7 for Cp and 2.0

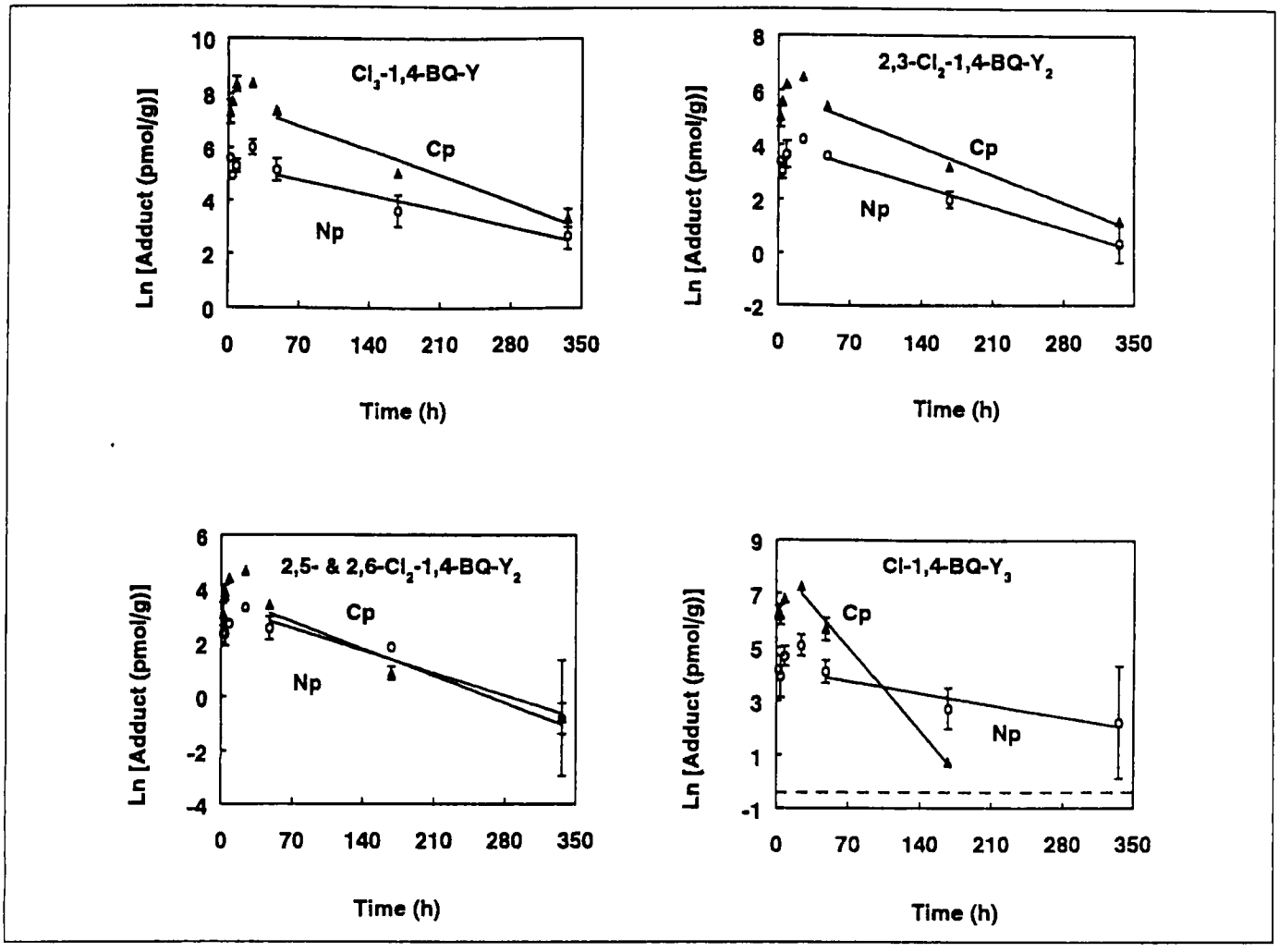

Figure 8. Stability of Cl_4-1,4-BQ-derived protein adducts in liver Cp and Np with time following administration of PCP to rats. (Mean values and standard deviations are shown for 3 animals per group. Solid lines represent least-squares regressions for the last three time points and dashed line represents 2/3 the level of the limit of detection.)

for Np, and in isolated proteins it was about 1.9 for Cp and 0.7 for Np. Since the ratio of monosubstituted to multisubstituted adducts was smaller for Cp in liver homogenate than in isolated proteins (i.e. 0.7 versus 1.9), we infer that reactions with small sulphydryl-containing species were more prevalent in the cytosol than were reactions with other proteins. On the other hand, this ratio for Np was larger in homogenates than in isolated proteins (2.0 versus 0.7) suggesting that multiple protein reactions were more prevalent in the nuclei.

When we incubated liver homogenates with either Cl_4-1,4-BQ or Cl_4HQ, similar rates of adduct production were observed (Table 3). This suggests that Cl_4HQ can undergo rapid oxidation to form Cl_4-1,4-BQ, even without the addition of NADPH. However, we detected no adducts of Cl_4-1,4-SQ in incubates of Cl_4HQ, despite previous evidence that this semiquinone was produced by Cl_4HQ *in vitro* without the presence of NADPH (Witte *et al.* 1985). Indeed, we only detected Cl_4-1,4-SQ-Y and Cl_4-1,2-SQ-Y in liver nuclei which had been incubated with PCP in the presence of NADPH.

When we investigated Cp and Np from rats to which PCP had been administered, we were able to detect both monosubstituted and multisubstituted adducts arising from Cl_4-1,4-BQ. In addition, we detected significant quantities of the semiquinone adducts, Cl_4-1,2-SQ-Y and Cl_4-1,4-SQ-Y. In a parallel experiment, we have measured these adducts in blood proteins and found that the levels of PCP-derived quinone and semiquinone adducts of Cp were generally much greater than those of blood proteins (Waidyanatha *et al.* 1996). The production of these adducts in liver Cp and Np was generally represented by a non-linear function of the dosage, with greater-than-proportional production of quinone adducts above 20 mg PCP per kg body weight (Figure 6) and less-than-proportional production of semiquinone adducts (Figure 7). These non-linear relationships point to saturable metabolism of PCP. Evidence suggests that the formation of quinols (Cl_4HQ and Cl_4CAT) from PCP is primarily mediated via microsomal cytochrome P450 and that the quinols then form the respective semiquinones via one-electron oxidations (van Ommen *et al.*

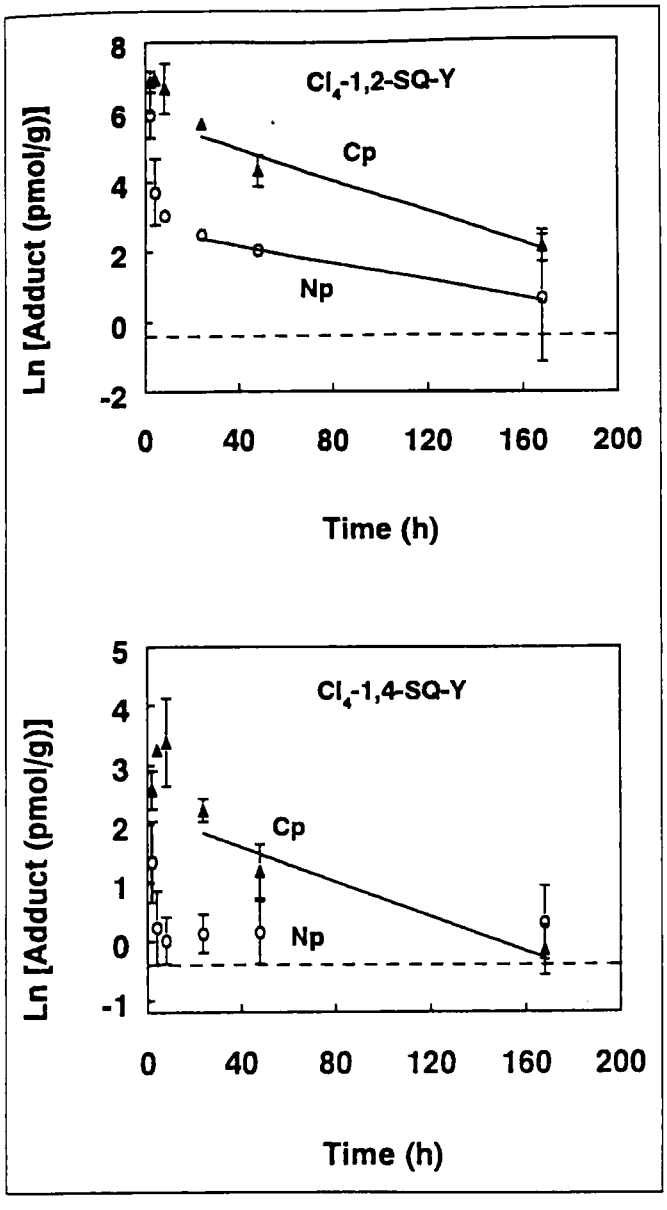

Figure 9. Stability of Cl_4-1,2-SQ- and Cl_4-1,4-SQ derived protein adducts in liver Cp and Np with time following administration of PCP to rats. No semiquinone adducts were detected at 336 h. (Mean values and standard deviations are shown for three animals per group. Solid lines represent least-squares regressions for the last three time points and dashed line represents 2/3 the level of the limit of detection.)

1988). It has also recently been reported that peroxidases can oxidize PCP to Cl_4-1,4-BQ without intermediate formation of Cl_4HQ (Samokyszyn *et al.* 1995). Our results tend to support the presence of both pathways. We speculate that, as the saturation of cytochrome P450 is achieved, the production of Cl_4HQ and Cl_4CAT diminishes with concomitant reduction in levels of the semiquinone adducts (Figure 7). This saturation of P450 enzymes then leads to conditions favouring the peroxidase pathway, with the resulting increase in the rate of production of adducts of Cl_4-1,4-BQ (Figure 6). Such behaviour suggests that the P450 pathway is of higher affinity for PCP but of lower capacity than the peroxidase pathway. We also recognize the possibility that these results may arise from two distinct isozymes of cytochrome P450 (Goeptar *et al.* 1995).

The relatively large amounts of multisubstituted adducts of Cl_4-1,4-BQ of liver proteins point to toxicological consequences. As noted previously, we suspect that these multisubstituted adducts contain both glutathione conjugates and protein–protein cross-links. The general toxicity of quinone-thioethers has long been recognized (Monks and Lau 1992), and Cl_4-1,4-BQ and its glutathione conjugates in particular have been shown to rapidly inactivate glutathione-S-transferase through covalent binding (van Ommen *et al.* 1991). Likewise, protein–protein cross-linking was found to be involved in the cytoskeletal alterations in rat hepatocytes induced by 1,4-BQ (Thor *et al.* 1988). Furthermore, the ability of Cl_4-1,4-BQ to produce protein–protein cross-links strongly suggests that it can also cross-link other nuclear macromolecules including DNA, with obvious toxicological implications.

A previous investigation of PCP metabolism in rat liver microsomes showed that Cl_4HQ was 1.5–6 times more abundant than Cl_4CAT (van Ommen *et al.* 1986). Nonetheless, significant quantities of Cl_4CAT were produced in that investigation and we expected to find adducts of both Cl_4-1,4-BQ and Cl_4-1,2-BQ in our study. Yet, we only detected Cl_4-1,2-BQ-derived adducts *in vitro* in untreated rat liver nuclei incubated with PCP, and did not detect any such adducts *in vivo*.

Adducts of Cl_4-1,4-BQ were much more abundant than those of Cl_4-1,4-SQ following metabolism of PCP, both in liver nuclei (about 20-fold more Cl_4-1,4-BQ-Y than Cl_4-1,4-SQ-Y, Table 4) and *in vivo* (about 160-fold more, Figures 6 and 7). However, for the 1,2-isomers of the quinone and semiquinone, adducts of Cl_4-1,2-SQ but not of Cl_4-1,2-BQ were found *in vivo* (Figure 7) and 15-fold more Cl_4-1,2-BQ than Cl_4-1,2-SQ was found *in vitro* (Table 4). These results suggest that liver nuclei, at least in part, contributed to the bioactivation of PCP and that the formation of Cl_4-1,2-BQ was impeded *in vivo* because of depletion of Cl_4-1,2-SQ, presumably due to reactions with nucleophiles. Given the substantial quantities of Cl_4-1,2-SQ-Y which we detected in these experiments we speculate that the presence of Cl_4-1,2-SQ within liver nuclei could lead to substantial DNA damage through covalent binding and/or to DNA strand breaks.

The stability of PCP-derived adducts was investigated both *in vitro* and *in vivo*. When adducted proteins were incubated at 37 °C *in vitro*, no degradation was observed (data not shown). However, investigations of Cl_4-1,4-BQ-modified liver homogenates showed pronounced losses of all adducts of Cp with first-order elimination rates of about 0.35 h^{-1} (Figure 5). We suspect that this instability arose primarily from continued reactions of the quinone adducts with small sulphydryl-containing molecules, notably glutathione. Likewise, all quinone adducts *in vivo* were eliminated over the course of the experiment with biphasic kinetics which included a rate constant of terminal phase of about 0.014 h^{-1} for Cp and 0.008 h^{-1} for Np (Figure 8). Since protein adducts can be eliminated *in vivo* by various processes, i.e. protein turn-over, random loss, and intrinsic instability (Fennel *et al.* 1992), we note that this terminal elimination rate constant for quinone adducts of Cp is more than twice the endogenous turn-over rate (0.0057 h^{-1}, Arias

et al. 1988), and probably reflects a significant contribution due to continued reactions with sulphydryl groups.

Since Cl_4SQ-Y should not be subject to the intrinsic instability experienced by the quinone adducts (due to additional substitution reactions), we expected the rates of elimination of the semiquinone adducts to be slower than their quinone counterparts *in vivo*. However, as indicated in Figure 9, elimination of both Cl_4-1,2-SQ-Y and Cl_4-1,4-SQ-Y proceeded at rates equal to or greater than those of the quinone adducts in the Cp fraction (0.015–0.023 h^{-1} for semiquinones versus 0.014 h^{-1} for quinones). This indicates that Cl_4SQ adducts are also being eliminated from Cp at rates much greater than the endogenous protein turnover rate, suggesting adduct instability. The same process of adduct loss appears to take place in Cl_4-1,2-SQ-Y of Np, with an elimination rate constant for the terminal phase of 0.013 h^{-1}, but not in Cl_4-1,4-SQ-Y, owing to the apparent stability of the semiquinone adducts during the period from 24–160 h post-exposure.

It is also worth noting that the similar times for adducts to reach peak concentration between Cp and Np (4 h in Cp versus 2 h in Np) imply that chlorinated benzosemiquinones and benzoquinones may arise from nuclear enzymes (presumably nuclear cytochrome P450) as well as from microsomes. The level of Cl_4-1,4-BQ-derived adducts produced by PCP metabolism in liver nuclei was about 0.02 times that produced by incubation with Cl_4-1,4-BQ (Tables 2 and 4), implying that the efficiency of conversion of PCP to Cl_4-1,4-BQ by isolated liver nuclei was about 2%.

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