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THE MICROSOMAL METABOLISM OF HEXACHLOROBENZENE

ORIGIN OF THE COVALENT BINDING TO PROTEIN

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Abstract—The microsomal metabolism of hexachlorobenzene is studied, with special attention to the covalent binding to protein. The metabolites formed are pentachlorophenol and tetrachlorohydroquinone. In addition, a considerable amount of covalent binding to protein is detected (250 pmoles pentachlorophenol, 17 pmoles tetrachlorohydroquinone and 11 pmoles covalent binding in an incubation containing 50 μmoles of hexachlorobenzene). In order to establish the potential role of reductive dechlorination in the covalent binding, the anaerobic metabolism of hexachlorobenzene was investigated. At low oxygen concentrations no pentachlorobenzene was detected, and only very small amounts of pentachlorophenol as well as covalent binding, indicating a relationship between covalent binding and the microsomal oxidation of hexachlorobenzene. Incubations with ^{14}C-pentachlorophenol at low concentrations showed that a conversion-dependent covalent binding occurs to the extent of 75 pmole binding per nmole pentachlorophenol. This is almost enough to account for the amount of label bound to protein observed in hexachlorobenzene incubations. This indicates that less than 10% of the covalent binding occurs during conversion of hexachlorobenzene to pentachlorophenol, and the remainder is produced during conversion of pentachlorophenol. The major product of microsomal oxidation of pentachlorophenol is tetrachlorohydroquinone, which is in redox-equilibrium with the corresponding semiquinone and quinone (chloranil). The covalent binding is inhibited by addition of ascorbic acid or glutathione to the hexachlorobenze incubations. Ascorbic acid decreases the covalent binding with a simultaneous increase in formation of tetrachlorohydroquinone, probably due to a shift in the redox-equilibrium to the reduced side. Glutathione does not act as a reducing agent, since the inhibition of covalent binding is not accompanied by an increase in tetrachlorohydroquinone formation. Instead, glutathione reacts with chloranil, producing at least three stable products, probably in a Michael-type reaction. These results strongly indicate the involvement of chloranil or the semiquinone radical in the covalent binding during microsomal hexachlorobenzene metabolism.

Hexachlorobenzene (HCB)‖ has been used worldwide as a fungicide and is formed as waste during the production of a number of chemicals. Although it is a hepatocarcinogen [1] it is perhaps best known for its disturbance of the hepatic porphyrin synthesis [2]. Evidence has accumulated that HCB has to be metabolized in order to become porphyrinogenic: induction of cytochrome P-450 by phenobarbital enhances the porphyrinogenic action [3–6], while selective inhibition of cytochrome P-450 in a primary chick embryo liver cell culture decreases its action [7]. We have previously investigated the microsomal metabolism of HCB, and shown that HCB is mainly converted to pentachlorophenol (PCP), while a small amount of tetrachlorohydroquinone (TCHQ) is also detected [8]. TCHQ has been reported to be a metabolite of PCP[9]. During the microsomal metabolism of HCB a substantial amount (±10%) of the total metabolites becomes covalently bound to microsomal proteins. Since the porphyrinogenic action of HCB is due to an inhibition of the liver enzyme uroporphyrinogen decarboxylase, it is tempting to assume that this inactivation is caused by a covalent modification as a result of HCB metabolism. These considerations led us to investigate in detail the origin of the covalent binding.

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‖ Abbreviations used: CAN, chloranil; HCB, hexachlorobenzene; PCB, pentachlorobenzene; PCP, pentachlorophenol; TCHQ, tetrachlorohydroquinone.

MATERIALS AND METHODS

Chemicals. U-^{14}C-Hexachlorobenzene (Amersham U.K. 70 mCi/mmole) contained 0.12% pentachlorobenzene (PCB) and 0.04% tetrachlorobenzene as radiochemical impurities. U-^{14}C-pentachlorophenol (CEA, Gif-sur-Yvette, France, 35 mCi/mmole) contained 2.2% tetrachlorophenol as radiochemical impurity, determined using the HPLC assays described below. ^{18}O-H_2O (99%) was from Amersham U.K. The origin of all other chemicals has been described elsewhere [8].

Microsomal incubations. Microsomes of male 12-week-old Wistar rats induced with 0.1% HCB for 14 days were prepared as described previously [8]. The standard incubation mixture contained 25 μM ^{14}C-HCB (3.5 μCi, in 50 μl acetone), 3 mM $MgCl_2$, 0.1 M

potassium phosphate-buffer pH 7.4 and 4 mg microsomal protein in a final volume of 2 ml. The reaction was started by addition of 1 mM NADPH. PCP-incubations contained 0.05–1 μM ^{14}C-PCP (3.75–15 nCi in 50 μl acetone). After 10 min incubation at 37°, the reaction was terminated with trichloroacetic acid (final conc. 3%). Total (>99.9%) soluble radioactivity was extracted with ether (2 × 3 ml). The extracts were dried with Na_2SO_4 and the ether was evaporated in a stream of nitrogen. Residues were dissolved in 50 μl of methanol for HPLC analysis. For anaerobic incubations, the incubation mixture was flushed with argon and degassed four times. HCB and NADPH were added by syringe through the seal, to start the reaction. Some anaerobic incubations also contained glucose oxidase (50 units) and glucose (10 mM). The oxygen concentration was measured in the gas-phase of tubes, treated similarly, but with non-radioactive HCB. One hundred microlitres of the gas-phase was injected onto a GLC equipped with a catharometer (Pye Unicam CDC chromatograph, molecular sieve 5A 80–100 mesh column 5ft × 4 mm I.D., carrier gas argon. To be certain of equilibrium between gas- and liquid-phase the tubes were vigorously shaken before measurement. This method showed a range in oxygen concentration between 280 and 1000 ppm, while addition of glucose/glucose oxidase resulted in an oxygen concentration of 100–500 ppm.

HPLC-analysis of metabolites. Separation of HCB, TCHQ, chloranil (CAN, tetrachloro-*p*-benzoquinone), PCP and PCB was achieved by injection of the sample in 50 μl of methanol (see above) plus 10 μl of methanol containing markers, on a Perkin Elmer series 4 HPLC equipped with a Chrompack lichrosorb 150 × 4.6 mm 5RP18 column, eluted with a gradient of 60% methanol in a 0.1% acetic acid solution in water to 100% methanol (1.67% per minute for 15 min followed by 1.5% per min for 10 min and kept at 100% for 5 min). Flow rate was 1.0 ml/min and detection was at 254 nm. One-millilitre fractions were collected and screened for radioactivity (5 ml atomlight scintillation fluid (NEN) in a Packard tricarb counter).

Covalent binding to protein. After ether extraction the aqueous (protein) samples were extracted with 3 × 10 ml methanol, 3 × 10 ml ethanol and 3 × 10 ml ether. After drying the protein pellets were dissolved in 1 ml Packard soluene 350 and 15 ml scintillation fluid and the amount of radioactivity was determined.

GC-MS-determination of ^{18}O-incorporation in PCP and TCHQ. Unlabelled HCB was incubated as described above for the radioactive compound, but in water containing 25% ^{18}O-H_2O. The ether extract was methylated with diazomethane, and PCP-methylether and TCHQ-dimethylether were isolated by HPLC (isocratic elution in 100% acetonitril, 0.5 ml/min on a Chrompack hypersil 290 × 4.6 mm 5 RP18 column, k' 2.0 and 1.7, respectively). After evaporation of the acetonitril the samples were dissolved in 5 μl of ether and injected into a GC-MS (VG MM7070). Samples were injected in a 3% OV-17/Chromosorb WHP column (2 mm I.D. × 1.5 m) using a temperature gradient of 160–200° at 6°/min. Selected ion recording was performed for peaks at m/z 277.86 and 279.86 (molecular ion peaks of PCP-methylether, further termed M^+ and $M^+ + 2$) and at m/z 258.89 and 260.89 (M^+ minus CH_3 peaks of TCHQ-dimethylether). The ratios between the integrated M^+ and $M^+ + 2$ signals were calculated and compared with the corresponding values found for the reference compounds. Both PCP- and TCHQ-methylether derived from incubations in unlabelled water and the synthetic compounds were measured as a reference.

RESULTS

Metabolism of HCB

As found previously [8], the only products of the microsomal oxidation of HCB are PCP (90% of total metabolites) and TCHQ (5–10% of metabolites), together with a small amount of material covalently bound to proteins. Of TCHQ, both the 1,2- and 1,4-isomers were detected, of which the 1,4-diol was the major fraction. The apparent K_m of the formation of both PCP and TCHQ was determined to be 34 μM

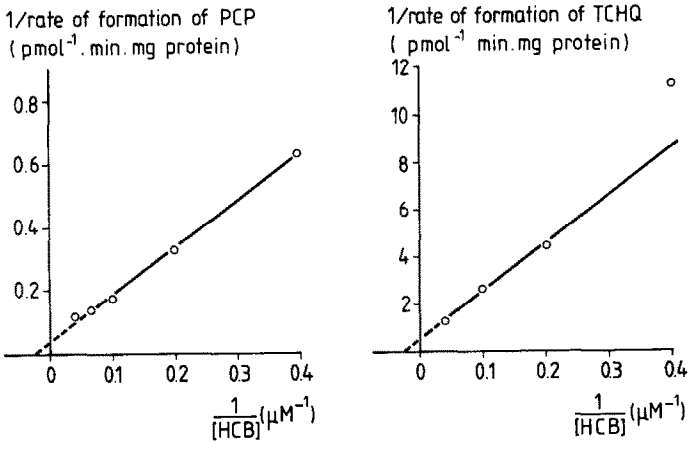

Fig. 1. Lineweaver–Burke plots of the formation of PCP and TCHQ in the microsomal oxidation of HCB. 25 μM ^{14}C-HCB was incubated for 10 min in a suspension of microsomes derived from male HCB-induced rats (2 mg protein/ml), in the presence of 1 mM NADPH. Products were separated from HCB by HPLC and quantitated by measurement of radioactivity.

Table 1. The microsomal metabolism of HCB

Incubations	Total conversion* (pmole)	PCP (pmole)	PCB (pmole)	TCHQ (pmole)	Covalent binding (pmole)
1 mM NADPH	260	220	0	14	15.2
1 mM NADH	35	0	0	4	0
1 mM NADPH + 1 mM NADH	265	225	0	20	7.3
Low oxygen Concentration	15–65	13–58	0	0	0–1.3

Twenty-five-micromolar ^{14}C-HCB was incubated at a protein concentration of 2 mg/ml. Microsomes were derived from male HCB induced rats. Incubations were performed in a volume of 2 ml at 37°. In the low oxygen concentration incubations oxygen was removed by repeated degassing and purging with argon in a sealed incubation tube. The upper and lower limit values of conversion at low oxygen pressure conditions are shown. Values in between show no PCB or covalent binding. The variation in metabolism under low oxygen conditions is presumably due to the variation in oxygen concentration (see Methods section).

* Total conversion denotes the total amount of extractable metabolites produced and is calculated from the total amount of radioactivity eluting from the HPLC before the substrate.

with a V_{max} of 24 pmoles PCP/mg protein/min and 1.9 pmoles TCHQ/mg protein/min (Fig. 1). The formation of TCHQ is linear with time during the 10 min incubation time, while the PCP-formation shows a slight deviation from linearity after 5 min.

Substitution of NADPH with NADH results in zero conversion (Table 1), while simultaneous addition of both cofactors results in the same rate of metabolism as compared to the incubations with NADPH alone.

Under anaerobic or very low oxygen conditions, resulting in a PCP-formation of 4–26% of that under aerobic conditions, the formation of PCP, PCB and covalent binding was measured. No PCB-formation was detected. Only at 26% PCP-formation was a small amount of covalent binding detected (12% of that under aerobic incubations, Table 1.) The variation in PCP-formation is probably due to the variation in oxygen concentration (100–1000 ppm in the gas phase, see Methods section).

As shown in Table 2, there is no evidence for incorporation of oxygen from ^{18}O-H_2O into PCP or TCHQ. ^{18}O-incorporation would result in a 2 Dalton increase of the molecular weight, resulting in a shift in the ratios of the peaks. For both PCP and TCHQ these ratios are unchanged after microsomal incubation of HCB in ^{18}O H_2O, as compared to an incubation in ^{16}O-H_2O or the synthetic reference. The theoretical values for the peak ratios if oxygen is derived completely from H_2O are also stated in Table 2.

Covalent binding by pentachlorophenol

Under normal incubation conditions the amount of covalent binding of radioactivity to protein resulting from metabolism of HCB is 10.6 ± 2.3 pmoles/4 mg protein (N = 8). In order to establish whether this covalent binding originates from an intermediate generated during the formation of PCP from HCB, or from binding of PCP or a conversion product of PCP, we have incubated ^{14}C-PCP under the same conditions as ^{14}C-HCB but at PCP-concentrations which are comparable to the amount of PCP formed

Table 2. Origin of the oxygen in PCP and TCHQ

Sample	PCP ($M^+ + 2/M^+ \times 100$)	TCHQ ($M^+ - 13/M^+ - 15 \times 100$)
a) ^{16}O-H_2O	156.3 ± 2.7 (N = 9)	127.6 ± 4.7 (N = 10)
b) 25% ^{18}O-H_2O	158.3 ± 1.6 (N = 7)	126.9 ± 3.6 (N = 3)
Synthetic reference	157.0 ± 1.0 (N = 4)	126.5 ± 0.9 (N = 10)
Expected value if oxygen is derived from H_2O*	189.6	160.4 (1 × ^{18}O) 193.4 (2 × ^{18}O)

Unlabelled HCB incubations were performed under normal conditions (a) and in a system containing 25% ^{18}O-labelled H_2O. PCP and TCHQ were purified by extraction and HPLC of their methyl ethers and analysed by GC-MS (selective ion recording). The extent of incorporation of ^{18}O was determined by comparing the ratio's for peaks at m/z = 277.9 and 279.9 for PCP-methylether (M^+ and $M^+ + 2$) and at m/z = 258.9 and 260.9 ($M^+ - 15$ and $M^+ - 13$) for TCHQ-dimethyl ether.

* The expected value is calculated as follows: in the case of 25% ^{18}O-labelling, 25% of each mass peak will show a 2 mass-unit shift, resulting in a 25% decrease of each individual original mass peak and addition of this value to the peaks two mass units higher.

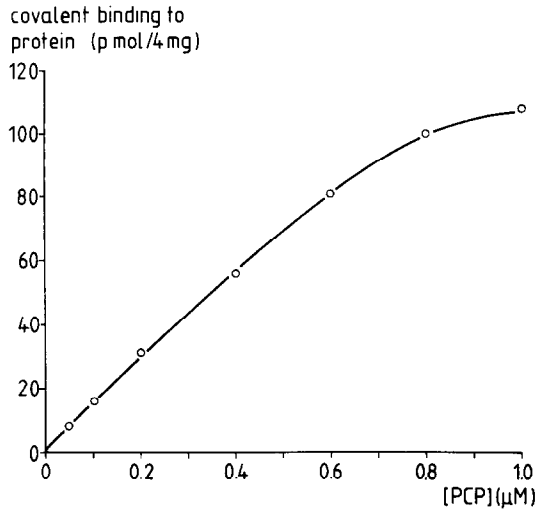

Fig. 2. Covalent binding to protein as a result of metabolism of PCP in microsomal incubations at very low PCP-concentrations. The values are corrected for binding of PCP itself. Covalent binding was measured by determining the amount of radioactivity in protein fractions of microsomal incubations after extensive washing with organic solvents.

during HCB-metabolism (0.250 ± 0.035 nmoles/4 mg protein/10 min in a 2 ml incubation; N = 8). The results are shown in Fig. 2. It appears that there is an almost linear relationship between the amount of covalent binding to protein and the PCP-concentration, with an initial slope of 75 pmoles binding/nmole PCP. Figure 2 represents the covalent binding due to conversion of PCP, since the values are corrected for the binding of PCP itself, which amounted to 18 pmoles binding per nmole PCP (derived from incubations with boiled microsomes and incubations without NADPH).

Inhibition of covalent binding

In the ^{14}C-HCB-incubations, ascorbic acid appeared to be able to completely inhibit the covalent binding at a concentration of 1 mM (Fig. 3). At concentrations which show no decrease in total conversion, there is a concentration-dependent decrease of covalent labelling. The loss of covalent binding is accompanied by an increase in TCHQ-formation: The concentration of TCHQ more than doubles after addition of 1 mM ascorbic acid to the incubation mixture. The amount of TCHQ which is produced due to addition of ascorbic acid is 60% of the amount of covalent binding which disappears. In some experiments, part of the TCHQ is measured as chloranil (CAN, tetrachloroquinone), due to partial oxidation of TCHQ during solvent removal. Glutathione also inhibits covalent binding (Fig. 3). As compared to ascorbic acid, glutathione shows a stronger inhibition: even at 0.01 mM an effect is observed. Up to 10 mM, the total conversion is not affected. In the case of glutathione, however, no increase in TCHQ is observed. Similar results are also obtained with cysteine: inhibition of covalent binding, but no increase in TCHQ.

To find an explanation for these phenomena, the reaction of glutathione with CAN was studied. Addition of glutathione to a solution of CAN in methanol at equimolar concentrations does not give rise to the formation of TCHQ, but results in the formation of at least three compounds, termed 1, 2 and 3, of which 3 is the major compound (Fig. 4). These compounds are different from TCHQ, CAN and glutathione in their absorption spectra and HPLC behaviour. All products are water-soluble. Upon reduction with ascorbic acid, fractions 1, 2 and 3 show a single absorption peak at 320, 330 and 335 nm, respectively. (TCHQ and CAN show single peaks at 310 and 286 nm, respectively.) Glutathione apparently does not act as a reducing agent towards the benzoquinone, but forms adducts.

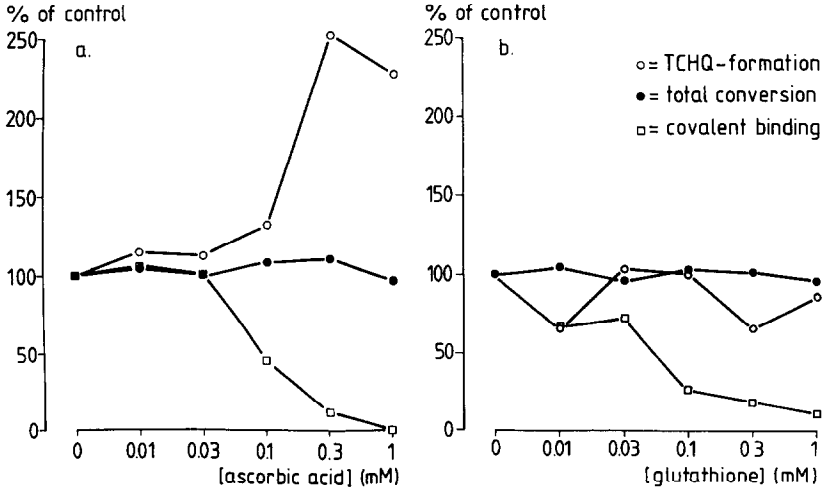

Fig. 3. The effect of ascorbic acid (a) and glutathione (b) on the covalent binding, total conversion and TCHQ-formation in the microsomal metabolism of HCB. Total conversion (the total amount of soluble metabolites produced) and TCHQ-formation were determined by HPLC of extracted substrate and products. Covalent binding was measured by counting the radioactivity of protein samples after extensive washing with organic solvents. All results are expressed as the percentage of activity with respect to a control incubation without ascorbic acid or glutathione.

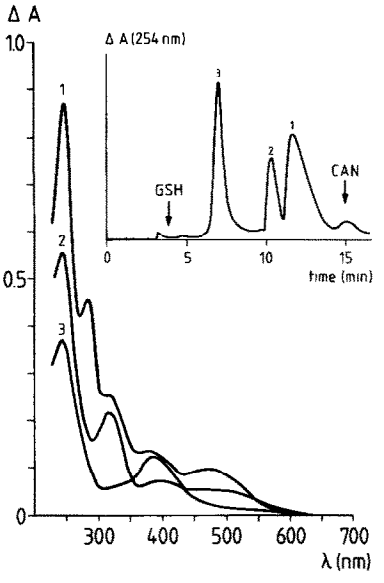

Fig. 4. HPLC-analysis and absorption spectra of the 3 products of the reaction of glutathione with chloranil. HPLC separation was achieved on a reversed phase C-18 column, eluted isocratically with 50% methanol and 50% H_2O containing 0.4% acetic acid. Spectra were recorded in the same solution.

DISCUSSION

HCB is a very poor substrate for the cytochrome P-450 reaction. The low V_{max}-value corresponds with the *in vivo* data on the slow biotransformation of HCB [10]. The HCB-concentration in the standard incubations [25 μM] is lower than the apparent K_m (34 μM). This is due to the very poor solubility of HCB. The near absence of metabolites and covalent binding in the anaerobic incubations indicate that a reductive dechlorination, as suggested by Renner [11], is unlikely. This implies that the covalent binding probably cannot be a result of the formation of a pentachlorophenyl radical, analogous to the radicals detected under anaerobic conditions in the microsomal metabolism of carbon tetrachloride and halothane [12, 13].

Our results indicate that the covalent binding which occurs during the microsomal metabolism of HCB does not originate from an intermediate which is generated during the turnover of HCB to PCP by cytochrome P-450, but results mainly from the conversion of PCP. The amount of PCP which is produced during HCB-conversion in our incubation system after 10 min is 250 pmoles, while the amount of covalent binding to microsomal protein is 10.6 pmoles. The ^{14}C-PCP incubations show that 114 pmoles PCP are necessary for 10.6 pmole covalent binding. Although the conversion of HCB to PCP is not completely linear during the 10 min incubation time, the average amount of PCP available for conversion and binding during the incubation time will not largely exceed half of the amount which is formed after 10 min, e.g. 125 pmoles PCP. This is 1.1 times the amount of PCP which produces the amount of covalent binding generated during HCB-turnover.

The major metabolite of PCP during metabolism by microsomes of HCB-induced male rats is TCHQ. The results of the microsomal incubation of HCB in ^{18}O-labelled H_2O show that both oxygen atoms of TCHQ are derived from molecular oxygen. This rules out the possibility that TCHQ is (partly) formed by H_2O-substitution of PCP or an intermediate during the PCP-formation, and indicates a sequential hydroxylation of HCB to PCP and of PCP to TCHQ by cytochrome P-450. TCHQ can undergo a two step–two electron oxidation to the benzoquinone-form (CAN), with a semiquinone radical as the one electron oxidation intermediate (Fig. 5). The existence of this semiquinone has been demonstrated by ESR and its lifetime was estimated to be 5 sec [14]. Either the tetrachloro-benzo-semiquinone radical might be responsible for the majority of the covalent binding which is measured after HCB metabolism (Fig. 5), or chloranil itself, which might undergo a Michael type addition. The finding that, together with the loss of covalent binding as a result of the presence of ascorbic acid, the TCHQ-concentration is raised by a comparable amount, seems to indicate the involvement of CAN or the benzoquinone radical in the covalent binding.

The protective action of ascorbic acid is probably due to a shift in the redox equilibrium to the reduced form (TCHQ), thus lowering the concentration of the semiquinone and CAN and causing a loss of covalent binding. The redoxpotentials (E_0) of the chloranil-TCHQ and the dehydroascorbic acid-ascorbic acid equilibria are 0.71 and 0.06 V, respectively [15], showing that 1 mM ascorbic acid is well capable of lowering the CAN concentration to zero levels. This also explains the rise of TCHQ concentration when the covalent binding is inhibited by

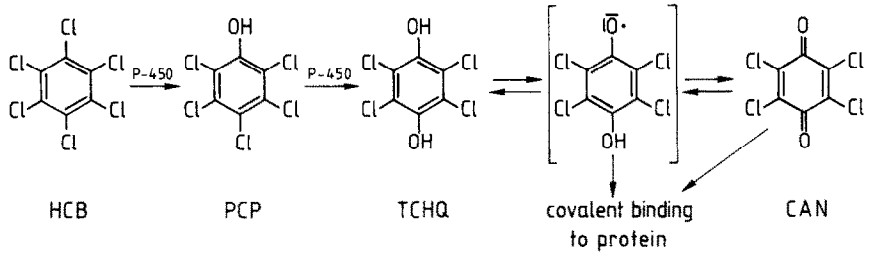

Fig. 5. Proposed route of microsomal metabolism of HCB and mechanism of covalent binding as a result of metabolism.

ascorbic acid. The protective role of ascorbic acid against HCB-induced porphyria in chick embryo liver cell culture has been reported earlier [3].

Glutathione and cysteine are known to react easily with benzoquinones. The reaction with 2,6-dichloro-p-benzoquinone for instance is used in the determination of cysteine [16]. In general, SH-groups react with the quinone in a Michael-type reaction followed by loss of HCl. From a reaction at equimolar amounts of glutathione and CAN, three products are formed, but no TCHQ is observed. In this way CAN might also react with protein-SH-groups. Glutathione and cysteine will act as inhibitors in this process. This explains the fact that, in contrast to ascorbic acid, no increase in TCHQ is measured at concentrations of glutathione and cysteine which completely inhibit covalent binding.

Diethylmaleate, a GSH-depleting agent, has been reported to enhance the porphyrinogenic action of HCB *in vivo* [4, 5]. The antiporphyrinogenic activity of ascorbic acid in an *in vitro* culture system and the observation that ascorbic acid and glutathione inhibit the covalent binding without inhibiting the cytochrome P-450 activity suggest the involvement of covalent binding in the porphyrinogenic action of HCB. This covalent binding is mainly due to pentachlorophenol metabolism.

On the other hand, pentachlorophenol has been shown to be unable to cause porphyria or induce cytochrome P-450 isoenzymes in rats [17–19]. We are presently investigating whether the lack of inducing capacity of PCP is responsible for its slow metabolism *in vivo* and lack of porphyrinogenic activity.

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