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Cytochrome P-450-Mediated Covalent Binding of Hexachlorophene to Rat Tissue Proteins

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SUMMARY

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Enzyme-mediated binding of radioactivity from hexachlorophene (2,2'-[^{14}C]methylenebis[3,4,6-trichlorophenol], [^{14}C]HCP) to rat hepatic microsomes occurred *in vitro* in the presence of NADPH and oxygen, and was inhibited by nitrogen, reduced glutathione, cysteine, piperonyl butoxide, and 2-diethylaminoethyl 2,2'-diphenylvalerate HCl (SKF 525-A). Binding was increased 2-fold by treatment of animals with phenobarbital (80 mg/kg intraperitoneally, daily for 3 days) and appeared to be dependent on the production of an active metabolite that was formed oxidatively in liver microsomes by a cytochrome P-450 mixed-function oxidase. Increased microsomal binding was not observed in animals treated with 3-methylcholanthrene (20 mg/kg intraperitoneally, daily for 2 days). Thin-layer chromatography of dinitrophenyl derivatives prepared from Pronase-digested microsomal protein and sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis of solubilized microsomes indicated that the radiolabeled products were covalently bound to amino acid residues of microsomal protein. Raney nickel desulfurization of Pronase hydrolysates released several HCP metabolites that were apparently bound to sulfur-containing amino acids such as cysteine and homocysteine. The enhancement in binding of [^{14}C]HCP to microsomal protein in the presence of epoxide hydrase inhibitors was consistent with the formation of HCP arene oxides or structurally related species. After intraperitoneal and oral administration of [^{14}C]HCP (5 mg/kg) to male rats treated with phenobarbital, radiocarbon was covalently bound mainly to proteins of the liver, plasma, and kidney. Covalently bound radioactivity in the liver was associated primarily with proteins in the microsomal and 105,000 × *g* supernatant fractions. Lesser amounts were present in the nuclear-cell debris and mitochondrial fractions.

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INTRODUCTION

Hexachlorophene (2,2'-methylenebis-[3,4,6-trichlorophenol]) has been used extensively in consumer products and in agriculture as an antibacterial and antifungal agent. Although HCP[2] has been

[2] The abbreviations used are: HCP, hexachloro-

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shown to be toxic to rats by either oral or intraperitoneal administration (1-3), little information is available on the mechanism(s) of toxicity.

We observed that the half-life of radioactivity from [^{14}C]HCP was quite long in the liver and various other tissues of the rat,[3] indicating that some HCP metabolites persist in the animal for appreciable times. Gandolfi *et al.* (4) reported that rat liver microsomes bind considerable amounts of radioactivity from [^{14}C]HCP. After incubation of [^{14}C]HCP with a complete microsomal system, over 89% of the radioactivity remained bound to washed microsomes, suggesting either a strong hydrophobic interaction or possible covalent binding to the microsomes. In a preliminary report, Corsini *et al.* (5) indicated that HCP covalently binds to liver microsomes *in vitro* and that binding was increased by previous phenobarbital treatment and inhibited by reduced glutathione, SKF 525-A, and carbon monoxide. HCP has also been shown to bind strongly to skin (6), bovine serum albumin[4] (7), the erythrocyte membrane (7), and various dehydrogenases.[5] Therefore the binding and resulting conformational changes may play an important role in the biological activity of the bisphenol.

The present study was initiated to assess further the role of mixed-function oxidases in the formation of HCP metabolites that bind covalently to cellular macromolecules, specifically rat tissue proteins.

MATERIALS AND METHODS

Reagents. Radioactive hexachlorophene (2,2'-[^{14}C]methylenebis[3,4,6-trichlorophenol]) (specific activity, 3.52 mCi/mmole) was purchased from New England Nuclear, and its radiochemical purity was established by thin-layer chromatography on silica gel plates developed in benzene–hexane–methanol–water (7:3:5:5 by volume). The specific activity was adjusted to 2.82 and 3.06 mCi/mmole with nonlabeled HCP that had been recrystallized from isopropyl alcohol–water prior to use. NADPH, 3-methylcholanthrane, and Pronase were obtained from Sigma Chemical Company. A generous sample of 2-diethylaminoethyl 2,2'-diphenylvalerate HCl (SKF 525-A) was kindly supplied by Dr. D. J. Reed, Department of Biochemistry and Biophysics, at this institution. Piperonyl butoxide, Raney nickel, 1,2-epoxy-3,3,3-trichloropropane, glycidol (2,3-epoxy-1-propanol), cyclohexene oxide (1,2-epoxycyclohexane), and 7,8-benzoflavone were purchased from Pfaltz and Bauer. Other chemicals used throughout this investigation were of analytical reagent grade.

Preparation of liver fractions. Adult male OSU Wistar rats (320–350 g), obtained from the Oregon State University Animal Resources Facility, were killed by cervical dislocation after CO_2 asphyxiation, and the livers were immediately removed, blotted free of excess liquid, weighed, minced, and then homogenized with a motor-driven glass-Teflon homogenizer in approximately 3 volumes of 1.15% KCl–0.01 M sodium phosphate buffer, pH 7.3–7.4. The nuclear-cell debris, mitochondrial, microsomal, and soluble enzyme fractions from control rats and animals treated with sodium phenobarbital in 0.9% NaCl solution or distilled water (80 mg/kg intraperitoneally, daily for 3 days) or 3-MC (20 mg/kg intraperitoneally, daily for 2 days) were isolated as described previously.[6] All rats were fasted for 12–24 hr prior to death. Protein concentrations were determined in duplicate according to Lowry *et al.* (8), using crystalline bovine serum albumin as the protein standard.

Determination of binding in vitro. The conditions of incubation and determination of binding have been described previously.[6] Briefly, a typical reaction mixture contained 0.062–0.125 mM [^{14}C]HCP (1.5–3.0 × 10^6 cpm), 0.05 or 0.1 M sodium

phene; 3-MC, 3-methylcholanthrene; TCA, trichloracetic acid; DNP, 2,4-dinitrophenyl; SDS, sodium dodecyl sulfate; ETCP, 1,2-epoxy-3,3,3-trichloropropane; FDNB, 1-fluoro-2,4-dinitrobenzene.

[3] D. R. Buhler, F. N. Dost, M. E. Rasmusson, and A. J. Gandolfi, manuscript in preparation.

[4] T. L. Miller and D. R. Buhler, manuscript in preparation.

[5] J.-L. Wang and D. R. Buhler, unpublished results.

[6] A. Miller III, M. C. Henderson, and D. R. Buhler, manuscript in preparation.

phosphate buffer (pH 7.3), 1 mM NADPH, and washed liver microsomes (4–5 mg of protein) in a total volume of 2 ml. Control flasks contained the above constituents but with no added cofactor. All reactions, unless stated otherwise, were conducted aerobically for either 30 min or 1 hr at 37° in a Dubnoff metabolic shaking incubator and terminated upon addition of 2 ml of 10% TCA. The total radioactivity in the control and reaction flasks was determined by counting aliquots in 15 ml of Aquasol (New England Nuclear), using a Packard Tri-Carb liquid scintillation spectrometer (model 3375). The TCA-insoluble precipitates were washed with TCA, extracted with ethyl ether (usually six or seven extractions), and redissolved in 1 N NaOH, and aliquots were counted after dark adaptation. Radioactivity was corrected for background and quenching (automatic external standardization) and converted to nanomoles of [^{14}C]HCP equivalent bound per milligram of protein after correction for non-cofactor-dependent or endogenous binding. Covalent binding refers only to NADPH-dependent or enzyme-mediated binding.

Examination of binding. Binding was further assessed by repetitive dissolution of ether-extracted microsomal preparations in 1 N NaOH, reprecipitation with TCA, and extraction with ethyl ether. Ether-extracted samples were also treated with 8 M urea or acid (2 N HCl), or digested with Pronase and allowed to react with 1-fluoro-2,4-dinitrobenzene as described by Jellinck *et al.* (9) and Jollow *et al.* (10). The dinitrophenyl derivatives were then sequentially extracted at pH 9 with ethyl ether and ethyl acetate, and at pH 1 with the latter solvent. All organic samples or extracts were dried over P_2O_5 under vacuum and dissolved in ethanol prior to radioassay.

Thin-layer chromatography of DNP derivatives. Thin-layer chromatography of the DNP derivatives was conducted essentially as described by Grant and Wicken (11). Aliquots (10–20 μl) were chromatographed on silica gel F_{254} plates (0.25 mm thick) and developed in the first dimension using a solvent system containing 1-butanol plus 0.15 N ammonium hydroxide (1:1, v/v, upper phase) described by Ghuysen *et al.* (12). Development in the second dimension was carried out with 1.5 M sodium phosphate buffer, pH 6 (13). Since the DNP derivatives are somewhat sensitive to light, all operations were carried out in the dark. The resolved DNP derivatives, visualized by their yellow color and ultraviolet light, were recovered from the plates and assayed for radioactivity in a liquid scintillation spectrometer. Binding was further evaluated by SDS–polyacrylamide disc gel electrophoresis as described below.

SDS–polyacrylamide disc gel electrophoresis. Solubilization of ether-extracted rat liver microsomes and SDS–disc gel electrophoresis were conducted as described previously.[6] The separating gel, 7% polyacrylamide, was buffered with Tris-HCl, pH 8.9; the stacking gel, 2.5% polyacrylamide, was buffered with Tris-HCl, pH 6.7; and the running buffer in the electrode chambers was Tris-glycine, pH 8.3, with 0.1% SDS. Electrophoresis was carried out at 3 mamp/gel for 90 min or until the tracking dye, bromphenol blue, reached within 1 cm of the lower end of the gel, and protein was visualized by staining with 1% Amido black, followed by destaining with 7% acetic acid. Molecular weights were estimated as described by Weber and Osborn (14). The logarithms of the molecular weights of known proteins were plotted against their respective mobilities, and the molecular weights of specific polypeptides were determined from the standard curve constructed (Fig. 1).

Epoxide hydrase inhibition and activation. The effects of 1,2-epoxy-3,3,3-trichloropropane and cyclohexene oxide, inhibitors of epoxide hydrase, and glycidol, an activator of epoxide hydrase (15), on the covalent binding of [^{14}C]HCP to microsomal protein were also examined. Increasing concentrations (1–10 mM) of ETCP and glycidol were added to reaction mixtures in 10-μl amounts from stock solutions prepared with spectrophotometric grade acetonitrile. Stock solutions of cyclohexene oxide were prepared with ethyl ether. The contents of all flasks were vigorously mixed immediately prior to the

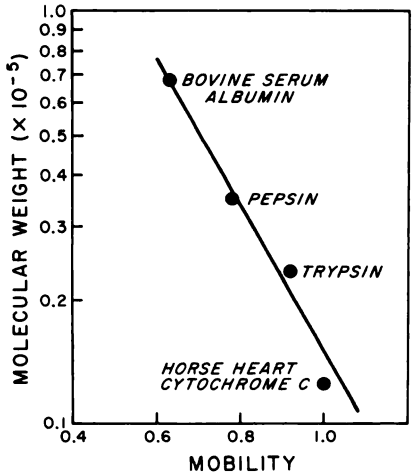

FIG. 1. *Molecular weight determinations by SDS-polyacrylamide disc gel electrophoresis*

The logarithms of the known molecular weights of the indicated proteins were plotted against their respective mobilities. Unknown molecular weights were determined from the standard curve.

addition of NADPH. Control mixtures (no NADPH) contained 10 μl of acetonitrile or ethyl ether. All mixtures were incubated aerobically at 37° for 30 min, and covalent binding was assessed as described above.

Binding in vivo. Covalent binding *in vivo* of radioactivity from [^{14}C]HCP in various selected tissues of the rat and in the subcellular fractions of the liver were also examined. Adult male rats (320–350 g), treated with phenobarbital (80 mg/kg intraperitoneally, daily for 3 days) and fasted for 12–24 hr prior to HCP treatment, received 5 mg/kg oral or intraperitoneal doses of [^{14}C]HCP (specific activity, 2.82 and 3.06 mCi/mmole) in 0.4 ml of corn oil. Animals were placed in individual metabolism cages, allowed water ad libitum but not fed, and killed after 3 and 6 hr for the intraperitoneal and oral doses, respectively. The various organs and tissues were removed, weighed, and homogenized with approximately 10 or 20 volumes of 1.15% KCl–0.01 M sodium phosphate buffer, pH 7.3–7.4. Liver homogenates were also subjected to differential centrifugation to obtain nuclear-cell debris, mitochondrial, microsomal, and soluble fractions as described previously.[6] Total tissue radioactivities were determined by counting aliquots of each homogenate following overnight dissolution in 1.0 N NaOH. Portions (1 ml) of each homogenate were also precipitated and washed with 10% TCA and treated as described for the binding analyses *in vitro*; i.e., they were extracted with ethyl ether and dissolved in NaOH, and aliquots were counted. The radioassays of all samples were performed in duplicate. Urine and feces were also collected from [^{14}C]HCP-treated animals for the determination of radioactivity.

Raney nickel treatment of Pronase digests. Samples of ether-extracted microsomal protein, recovered from binding experiments *in vivo* and *in vitro* conducted as described above, were digested with Pronase (80–100 units) for 48 hr at 37° and then lyophilized. Raney nickel desulfurization of the Pronase hydrolysates was conducted essentially as described by Corbett and Nettesheim (16). Portions (0.1 g) of the lyophilized preparations were extracted three times with ether, dissolved in phosphate buffer (30 ml), and added to 100-ml round-bottomed flasks containing 3 g of thoroughly washed nickel. Approximately 1.5 ml of 1 N HCl were added, and the mixtures were heated at 110–115° in an oil bath for 30 min. The mixtures were extracted with ether and dried over anhydrous Na_2SO_4, and excess solvent was evaporated using a rotary evaporator. Aliquots of the ether extract were assayed for radioactivity and chromatographed on silica gel F_{254} plates (0.25 mm thick). The solvent system consisted of benzene–hexane–methanol–water (7:3:5:5 by volume). Metabolites were visualized by iodine vapor and assayed for radioactivity.

RESULTS

Binding of [^{14}C]HCP to microsomal protein. Some requirements for the enzyme-mediated or covalent binding of radioactivity from [^{14}C]HCP to rat hepatic microsomes *in vitro* are given in Table 1. Binding was inhibited by 82.5% in a nitrogen atmosphere but was not significantly affected by NaCN (1 mM). Non-cofactor-dependent or endogenous binding, 0.5–0.8% of the added radioactivity or 0.3 nmole of

TABLE 1

Conditions for covalent binding of radioactivity from [^{14}C]hexachlorophene to rat liver microsomes in vitro

Rat liver microsomes (4–5 mg of protein) from phenobarbital-treated animals were incubated at 37° for 1 hr with 1 mM NADPH and 0.125 mM [^{14}C]HCP as described in MATERIALS AND METHODS. Results are the means of duplicate determinations. All values were corrected for non-cofactor-dependent or endogenous binding.

Reaction mixture	[^{14}C]HCP bound
	nmoles/mg protein/hr
Experiment 1	
Complete	1.49
Complete, heated (90–95°) microsomes	0.00
−NADPH	0.00
+N_2	0.26
Experiment 2	
Complete	1.84
−NADPH	0.00
+NaCN (1 mM)	1.88

[^{14}C]HCP equivalent per milligram of protein, occurred in the absence of either NADPH or oxygen. The enzymatic nature of the binding reaction was evident from the complete absence of cofactor-dependent binding when the microsomes were heated at 90–95° for 10 min prior to incubation.

Effect of [^{14}C]HCP and microsomal concentrations on binding. The effect of [^{14}C]HCP concentration on the covalent binding *in vitro* of radioactivity to washed rat liver microsomes from phenobarbital-treated animals is shown in Fig. 2. Binding increased with the concentration of [^{14}C]HCP, and maximal binding was obtained with 0.125 mM [^{14}C]HCP. The reduction in binding at the higher concentrations of HCP could have resulted from either a limited mixed-function oxidase-dependent activation of HCP and/or saturation of available binding sites.

A double-reciprocal plot of mixed-function oxidase-dependent binding of radioactivity from [^{14}C]HCP to microsomes is shown in Fig. 3. The apparent K_m for binding to microsomes from phenobarbital-treated animals was 20 μM, and the V_{max} was 0.48 nmole bound per milligram of protein per minute. These values are in agreement with data previously reported for the binding of acetaminophen (17) and

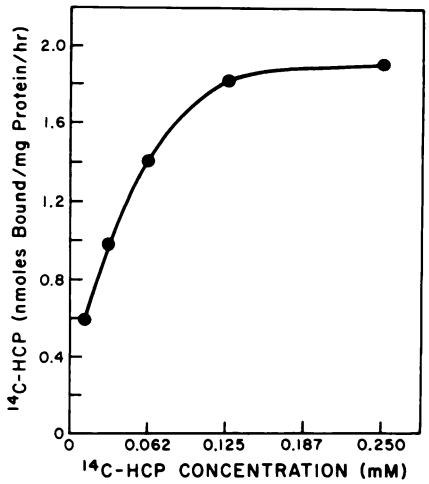

FIG. 2. *Effect of concentration on binding of radioactivity from [^{14}C]HCP to rat liver microsomes in vitro*

Microsomes (6 mg of protein) from phenobarbital-treated rats were incubated aerobically at 37° for 1 hr with 1 mM NADPH and [^{14}C]HCP (0.015, 0.031, 0.062, 0.125, and 0.25 mM) as described in MATERIALS AND METHODS.

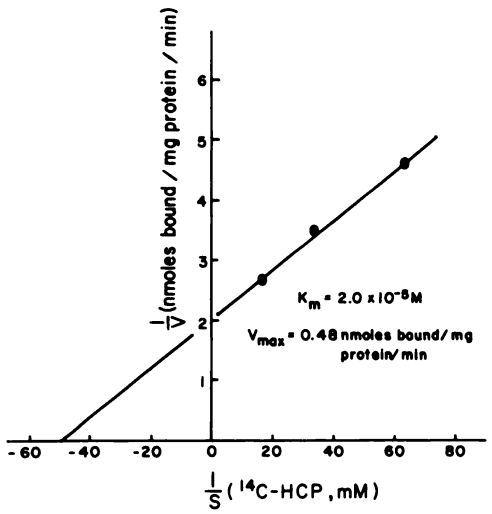

FIG. 3. *Lineweaver-Burk plot of mixed-function oxidase-dependent binding of [^{14}C]HCP to liver microsomes in vitro*

Microsomes (2–4 mg of protein) from phenobarbital-treated rats were incubated at 37° for 10 min with 1 mM NADPH and [^{14}C]HCP (0.016, 0.031, and 0.063 mM). Covalent binding was determined as described in MATERIALS AND METHODS.

furosemide (18) to mouse hepatic microsomes.

The effect of microsomal protein concentration on binding of radioactivity from [^{14}C]HCP to washed liver microsomes is shown in Table 2. Maximal binding, 7.1–7.2 nmoles/incubation mixture, occurred with 2 and 4 mg of microsomal protein in the reaction mixtures. Therefore, unless stated otherwise, all subsequent reactions *in vitro* were carried out using 4 mg of microsomal protein.

Effects of prior treatments on binding. Covalent binding of radiocarbon from [^{14}C]HCP by microsomes obtained from phenobarbital- or 3-MC-treated male rats was compared with binding by microsomes from control animals. Phenobarbital treatment increased binding approximately 2-fold, whereas no significant increase, other than slight stimulation in some cases, was observed with 3-MC (Table 3).

Inhibition of binding to microsomal protein. The effects of various inhibitors on the covalent binding of [^{14}C]HCP to rat liver microsomal protein are given in Table 4. In the presence of washed microsomes (4–5 mg) and NADPH (1 mM), cysteine (0.5 mM) and GSH (0.5 mM) inhibited binding by 8% and 28%, respectively. When the concentration was increased to 10 mM, cysteine and GSH inhibited binding by 56% and 67%, respectively. No appreciable inhibition of binding was observed in the presence of NaF (0.1 M). Piperonyl butoxide, which forms a stable complex with and inactivates cytochrome

TABLE 2

Effect of microsomal protein concentration on covalent binding of radioactivity from [^{14}C]hexachlorophene to washed liver microsomes

Increasing amounts of rat liver microsomes were incubated at 37° for 1 hr with 1 mM NADPH and 0.062 mM [^{14}C]HCP as described in MATERIALS AND METHODS.

Total microsomal protein	Radioactivity bound	
mg	*nmoles/mg protein/hr*	*nmoles/incubation vessel*
2	3.55	7.1
4	1.80	7.2
8	0.84	6.7
10	0.55	5.5
16	0.25	4.0
20	0.09	1.8

TABLE 3

Effects of prior treatments on maximal covalent binding of radioactivity from [^{14}C]hexachlorophene to rat liver microsomal protein in vitro

Liver microsomes (4–5 mg of protein) were incubated at 37° with 1 mM NADPH and 0.125 mM [^{14}C]HCP as described in MATERIALS AND METHODS. Treated animals received phenobarbital (80 mg/kg intraperitoneally, daily for 3 days) or 3-MC (20 mg/kg intraperitoneally, daily for 2 days). All animals were fasted for 12–24 hr prior to death. Values represent means ± standard deviations of at least five incubations, using microsomes from three to five rats.

Treatment	[^{14}C]HCP bound
	nmoles/mg protein/hr
None	0.76 ± 0.23
Phenobarbital	1.57 ± 0.32
3-MC	0.73 ± 0.19

TABLE 4

Inhibition of covalent binding of radioactivity from [^{14}C]hexachlorophene to rat hepatic microsomes in vitro

Rat liver microsomes (4–5 mg of protein) from phenobarbital-treated animals (80 mg/kg intraperitoneally, daily for 3 days) were incubated at 37° for 1 hr with 1 mM NADPH and 0.031–0.125 mM [^{14}C]HCP as described in MATERIALS AND METHODS.

Reaction mixture	[^{14}C]HCP bound	Inhibition
	nmoles/mg protein/hr	*%*
Experiment 1		
Complete	1.49	0.0
+Cysteine (0.5 mM)	1.37	8.1
+Cysteine (10.0 mM)	0.66	56.0
+GSH (0.5 mM)	1.07	28.1
+GSH (10.0 mM)	0.50	67.0
Experiment 2		
Complete	1.06	0.0
+Piperonyl butoxide (1 mM)	0.16	84.9
+NaF (0.1 M)	0.95	10.4
Experiment 3		
Complete	1.46	0.0
+7,8-Benzoflavone (0.1 mM)	0.64	56.2
+7,8-Benzoflavone (0.01 mM)	1.35	7.6
Experiment 4		
Complete	1.90	0.0
+SKF 525-A (1 mM)	0.20	89.4

P-450, inhibited binding by 85%, and SKF 525-A inhibited oxidative metabolism by approximately 90%. Binding was also inhibited by 56.2% and 7.6% in the presence of 0.1 mM and 0.01 mM 7,8-benzoflavone, respectively. Since the binding of radioactivity from [^{14}C]HCP was inhibited by sulfhydryl compounds, increased by prior phenobarbital induction of the hepatic microsomal enzymes, and decreased by inhibitors such as piperonyl butoxide 7,8-benzoflavone, and SKF 525-A, an active metabolite of HCP apparently was formed by enzymes of the cytochrome P-450 mixed-function oxidase system.

Covalent nature of binding. More than 92% of the radioactivity from [^{14}C]HCP was removed from microsomes after TCA precipitation, washing twice with TCA, and extraction six or seven times with ethyl ether. Additional amounts of bound radioactivity were removed by a more complete extraction involving repetitive precipitation and washing of liver microsomes with TCA followed by solubilization in 1 N NaOH (six times), treatment with 8 M urea and extraction with ethyl ether (twice), and adjustment to pH 1 with HCl and extraction with ethyl ether (twice). However, approximately 0.11% of the radioactivity added in the absence of NADPH and 1.74% of that added to the complete reaction mixture remained bound to microsomes after disruption of the secondary and tertiary protein structure by this extensive precipitation and extraction process. Significant amounts of radioactivity from [^{14}C]HCP also remained associated with liver proteins following prolonged dialysis against several changes of methanol. These results indicated that a small but significant amount of HCP metabolite(s) was covalently bound to rat liver microsomes *in vitro* (approximately 1.5 nmoles/mg of protein). Similar values for microsomal binding have been observed with other chemicals or drugs[6] (10, 19).

Further evidence of the covalent nature of [^{14}C]HCP binding was obtained from experiments in which the primary structure of liver proteins was broken by Pronase digestion and the resulting amino acids and peptides were then derivatized with FDNB. Enzymatic hydrolysis of exhaustively extracted liver proteins apparently released additional amounts of bound HCP metabolites, since 31–44% of the residual radioactivity in the controls (i.e., no FDNB) was recovered in the various extract fractions (Table 5). Nevertheless, most of the radioactivity from [^{14}C]HCP remained in the aqueous fraction. Much of this radioactivity (42–47%),

TABLE 5

Distribution of radioactivity from [^{14}C]hexachlorophene after Pronase digestion of liver protein, treatment with FDNB, and sequential extraction

Fraction and exposure	Treatment	Bound radioactivity			
		Ethyl ether, pH 9	Ethyl acetate, pH 9	Ethyl acetate, pH 1	Aqueous residue
		% original			
Homogenate[a]	Ethanol	14.7	6.7	22.8	55.7
Homogenate[a]	FDNB + ethanol	19.5	15.6	42.0	22.9
Microsomes *in vivo*[b]	Ethanol	9.7	2.8	20.2	67.2
Microsomes *in vivo*[b]	FDNB + ethanol	21.6	5.0	42.3	31.1
Microsomes *in vitro*[c]	Ethanol	9.6	4.3	17.0	69.0
Microsomes *in vitro*[c]	FDNB + ethanol	11.3	8.1	46.9	33.7

[a] Portions (1 ml) of liver homogenate from phenobarbital-treated animals injected intraperitoneally with 5 mg/kg of [^{14}C]HCP were extracted six times with ethyl ether, digested with Pronase, and treated with FDNB as described in MATERIALS AND METHODS.

[b] Hepatic microsomes from phenobarbital-treated animals injected intraperitoneally with 5 mg/kg of [^{14}C]HCP were treated as described above and in MATERIALS AND METHODS.

[c] Hepatic microsomes from phenobarbital-treated animals were recovered from reactions *in vitro* and treated as described above.

however, was removed in the pH 1 ethyl acetate extract after treatment of the digests with FDNB. Since FDNB reacts with the amino groups of amino acids and peptides to form DNP derivatives that are extractable with organic solvents under acidic conditions, the results indicated that the active metabolite of hexachlorophene was covalently attached to amino acid residues of microsomal protein.

Two-dimensional thin-layer chromatography of the DNP-amino acids or DNP-peptides from hepatic proteins substantiated the covalent nature of the binding reaction. Approximately 25 DNP derivatives were separated but not identified, and radioactivity was shown to migrate with several of the major spots (Fig. 4). Since appreciable radiocarbon (320 cpm) was recovered from the residual silica gel of the entire plate after visualization and elution of the DNP derivatives, additional radioactive spots may have been present but were too faint to detect under ultraviolet light.

SDS-polyacrylamide disc gel electrophoresis. SDS-disc gel electrophoresis of solubilized, ether-extracted microsomal control and reaction mixtures following incubations *in vitro* resolved 25–30 protein bands upon staining with 1% Amido black and destaining with 7% acetic acid. Comparison of electrophoretograms and radioactive profiles of the sectioned gels revealed at least two major radioactive areas in the reaction gel that corresponded with specific bands or peaks in the densitometer curves (Fig. 5). The finding that a radioactive HCP metabolite(s) and a particular polypeptide(s) migrated together in an electrical field again substantiates the existence of a strongly bound complex. The estimated molecular weights of the two major components indicated in Fig. 5 were 18,500–19,500 and 54,500–60,000, respectively.

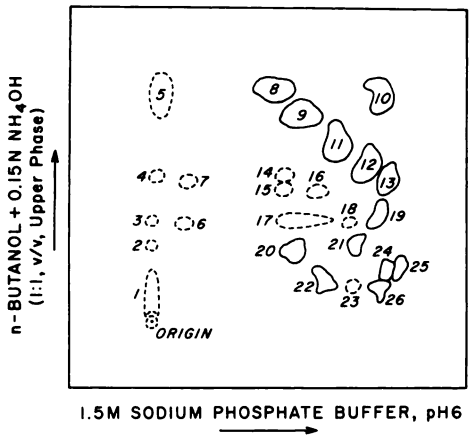

FIG. 4. *Tracing of two-dimensional thin-layer chromatographic separation of DNP derivatives prepared from Pronase-digested liver microsomal protein*

Radioactivity was primarily localized in the following spots: 1 (985 cpm, 44.6%), 2 (179 cpm, 8.1%), 3 (152 cpm, 6.9%), 4 (92 cpm, 4.2%), 5 (200 cpm, 9.1%), and 14 (105 cpm, 4.8%). The remaining spots and residual silica gel contained a total of 176 cpm and 320 cpm, or 7.9% and 14.5% of the added radioactivity, respectively.

Binding in vivo. The distribution and covalent binding *in vivo* of radioactivity from [^{14}C]HCP in various tissues of adult male rats treated with phenobarbital and then either intraperitoneally or orally with 5 mg/kg of the bisphenol are shown in Table 6. Radiocarbon was localized primarily in the liver, kidney, and plasma. The covalent binding of HCP metabolites *in vivo* was appreciably less than the binding observed with liver microsomes *in vitro* (Table 1). Approximately 65% and 76% of administered radioactivity was accounted for or recovered in the tissues, urine, and feces of rats treated intraperitoneally and orally with [^{14}C]HCP, respectively. Limited radiocarbon was excreted in the urine (1.7–3.2%) and feces (0.02–0.10%) in the short time periods employed in this study (Table 6). However, the distribution of tissue radioactivity and excretion data were similar to those reported by Buhler *et al.*[3] for rats at comparable times after administration of [^{14}C]HCP.

Subcellular location of bound radioactivity. The subcellular distribution of covalently bound radioactivity from [^{14}C]HCP in rat liver is shown in Table 7. After intraperitoneal and oral administration of [^{14}C]HCP, the major portion of the bound radioactivity was localized primarily in the microsomes (37–38%) and in the cytosol (25–27%). Lesser amounts of radioactivity were associated with the proteins of

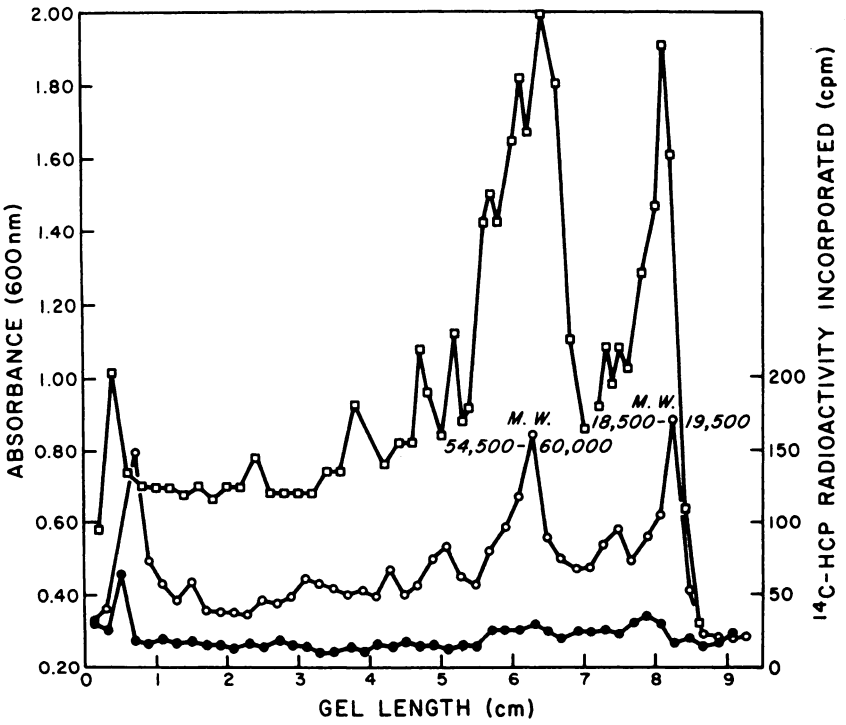

FIG. 5. *Densitometric curve* (□——□) *and radioactive profiles after SDS-polyacrylamide disc gel electrophoresis*

Ether-extracted hepatic microsomes were incubated with 0.125 mM [^{14}C]HCP in the absence (●——●) and presence (○——○) of 1 mM NADPH as described in MATERIALS AND METHODS.

the nuclear-cell debris and mitochondrial fractions.

Raney nickel desulfurization. In an attempt to determine the binding site(s) and assess the nature of the bound metabolite(s) of HCP, hepatic microsomes obtained from experiments *in vivo* and *in vitro* were precipitated and washed with TCA, extracted six times with ether, and digested with Pronase as described in MATERIALS AND METHODS. The resultant digests were lyophilized, extracted three times with ether, and subjected to Raney nickel desulfurization.

Raney nickel treatment released 13–20% of the covalently bound HCP metabolites that were extractable with ethyl ether. Thin-layer chromatography of aliquots of the ether extract revealed a minimum of five or six radioactive spots. The R_F values of the cleaved metabolites were 0.17, 0.23, 0.38, 0.41, and 0.94 in benzene-hexane-methanol-water (7:3:5:5 by volume). An additional polar component remained at the point of application. A considerable amount (63%) of released radiocarbon was extracted into 0.1 N NaOH from the ether extract, and the presence of phenols and/or acids was indicated. Acidic treatment and ether extraction prior to desulfurization removed approximately 4–6% of the radioactivity associated with the protein digests. Since Raney nickel selectively cleaves carbon-sulfur bonds, the HCP metabolites released from the Pronase digests were probably attached to the sulfur-containing amino acids (e.g., cysteine and/or homocysteine) via a carbon-sulfur bond (18). However, a significant portion (40%) of the bound radiocarbon remained in the aqueous fraction after Raney nickel treatment and solvent extraction, indicating possible attachment of HCP metabolites to other binding sites, such as the NH_2 groups of lysine. More than 80% of the radioactivity associated with the Pronase

digests was recovered in the various fractions analyzed.

Epoxide hydrase inhibition and activation. To evaluate further the nature of the bound HCP metabolites, microsomal oxidations were conducted *in vitro* in the presence of inhibitors and an activator of epoxide hydrase that converts arene oxides to less reactive dihydrodiols (15). The effects of ETCP, cyclohexene oxide, and glycidol on the binding of radioactivity from [^{14}C]HCP to rat hepatic microsomes are shown in Table 8. Covalent binding was increased substantially (40–58%) in the presence of 1.0 mM, 2.5 mM, and 5.0 mM ETCP. Enhanced binding was also observed with cyclohexene oxide at the same concentrations. The inhibition of the enzymatic hydration of an HCP epoxide by ETCP and cyclohexene oxide apparently increased the steady-state level of the epoxide, resulting in significant increases in covalent binding (18). In addition to the apparent inhibition of epoxide hydrase, the decreases in binding noted at 10 mM ETCP and cyclohexene oxide indicated a reduction in liver monooxygenase activity, which may have limited the formation of the arene oxide(s). Slight inhibition of the binding of radioactivity from [^{14}C]HCP to microsomal protein was observed when microsomal oxidations were conducted in the presence of high concentrations of glycidol, an activator of epoxide hydrase (Table 8). Acetonitrile and ethyl ether (10 μl), added to all control and reaction mixtures, appeared to have little effect on the degree of binding.

TABLE 6

Covalent binding of radioactivity in tissues of rats after oral and intraperitoneal administration of [^{14}C]hexachlorophene

Radioactivity bound to TCA-insoluble, ether-extracted fractions was determined. Values are means of duplicate determinations.

Organ or tissue	Bound radioactivity	
	Intraperitoneal dose[a]	Oral dose[b]
	nmole HCP equivalent/mg protein	
Brain	0.001	0.000
Fat	0.006	0.001
Heart	0.002	0.003
Kidney	0.035	0.030
Liver	0.076	0.040
Lung	0.005	0.004
Muscle	0.003	0.001
Plasma	0.045	0.056
Spleen	0.007	0.001
Testes	0.002	0.003

[a] Phenobarbital-treated animals received 5 mg/kg of [^{14}C]HCP (specific activity, 3.06 mCi/mmole) and were killed 3 hr later. Urine and feces contained 1.70% and 0.02% of the dose, respectively.

[b] Phenobarbital-treated animals received 5 mg/kg of [^{14}C]HCP (specific activity, 2.82 mCi/mmole) and were killed 6 hr later. Urine and feces contained 3.17% and 0.10% of the dose, respectively.

TABLE 7

Subcellular distribution of covalently bound radioactivity from [^{14}C]hexachlorophene in rat liver after oral and intraperitoneal administration

Rats treated with phenobarbital received 5 mg/kg of [^{14}C]HCP (specific activity, 2.82–3.06 mCi/mmole). Intraperitoneally and orally treated animals were killed at 3 and 6 hr, respectively. Radioactivity was determined in TCA-insoluble, ether-extracted fractions. Values are means of duplicate determinations.

Cell fraction	Intraperitoneal dose			Oral dose		
	Total HCP per fraction	HCP per mg of protein	Radioactivity covalently bound	Total HCP per fraction	HCP per mg of protein	Radioactivity covalently bound
	nmoles HCP equivalent		*% total fraction*	*nmoles HCP equivalent*		*% total fraction*
Nuclear + cell debris	21.0	0.041	15.6	17.5	0.025	17.7
Mitochondria	13.8	0.030	18.6	8.78	0.028	19.5
Microsomes	21.5	0.10	38.1	15.0	0.053	37.2
Soluble (105,000 × *g* supernatant)	51.8	0.074	27.7	28.5	0.036	25.7

TABLE 8

Effects of 1,2-epoxy-3,3,3-trichloropropane, cyclohexene oxide, and glycidol on binding of radioactivity from [^{14}C]hexachlorophene to rat hepatic microsomes in vitro

Washed rat liver microsomes (2–4 mg of protein) were incubated at 37° for 30 min with [^{14}C]HCP (0.031–0.062 mM) as described in MATERIALS AND METHODS. Values are the means ± standard deviations of duplicate analyses from at least three animals and were corrected for non-cofactor-dependent binding of [^{14}C]HCP. ETCP and glycidol were added to incubation mixtures in acetonitrile (10 μl). Cyclohexene oxide was added in ethyl ether (10 μl).

Inhibitor or activator	Concentration	[^{14}C]HCP bound	
	mM	*nmoles/mg protein/30 min*	*% complete reaction*
ETCP	0.0	1.33 ± 0.15	100
	1.0	1.87 ± 0.29	141
	2.5	2.10 ± 0.19	158
	5.0	1.96 ± 0.03	149
	10.0	1.51 ± 0.27	114
Cychlohexene oxide	0.0	1.12 ± 0.17	100
	1.0	1.26 ± 0.20	112
	2.5	1.58 ± 0.48	139
	5.0	1.52 ± 0.41	134
	10.0	1.05 ± 0.49	91
Glycidol	0.0	0.895 ± 0.18	100
	1.0	0.878 ± 0.11	99
	2.5	0.957 ± 0.24	106
	5.0	0.857 ± 0.34	94
	10.0	0.719 ± 0.20	80

DISCUSSION

HCP was oxidized via a cytochrome P-450 mixed-function oxidase system to reactive metabolites which then covalently bound to microsomal proteins. Binding required NADPH and oxygen and was inhibited by nitrogen, piperonyl butoxide, 7,8-benzoflavone, and SKF 525-A (Tables 1 and 4). Cysteine and GSH also inhibited covalent binding when included in the microsomal reaction mixtures, but appeared to be less effective at lower concentrations (0.5– 1 mM) than other chemicals and drugs[6] (19). The conjugation of reactive HCP metabolites with GSH did not appear to be a significant or major pathway in the oxidative metabolism of the bisphenol *in vitro*. Similar results were reported by Selander *et al.* (20) for the conjugation of the arene oxides of chlorobenzene with glutathione *in vitro*. The electrophilic reactants may not be readily accessible to the tripeptide, and, even under optimal reaction conditions, other pathways may be preferred. Under our experimental conditions, only a slight increase in GSH inhibition was observed when cytosol (105,000 × *g* supernatant) from uninduced animals was added to the microsomal incubation mixtures. Boyland and Williams (21) demonstrated that several epoxy compounds, including the polychlorinated insecticide dieldrin, were not substrates for the GSH transferases in liver supernatant preparations from rats. Apparently some epoxides react with GSH in the absence of transferase, others do not react even in the presence of enzyme, and some form conjugates only if the enzyme is present (22).

Treatment of animals with phenobarbital increased binding approximately 2-fold (Table 3), but 3-MC was only slightly stimulatory in some cases. Although phenobarbital and 3-MC enchance the metabolism of a variety of drugs and chemicals, they induce different hepatic cytochromes P-450 (23). The data suggested that a cytochrome P-450 rather than a cytochrome P_1-450 or P-448 pathway was predominantly involved in the activation of HCP.

The enchancement of binding in the presence of ETCP and cyclohexene oxide and the limited inhibition observed with glycidol supported the conclusion that arene oxides are initially formed in the metabolic activation of HCP by the cytrochrome P-450 monooxygenase system. The HCP epoxides may react directly with microsomal proteins, or they may be intermediates that are further metabolized or rearranged to other electrophilic species that subsequently bind to cell constituents.

These results confirm and extend the preliminary observations of Corsini *et al.* (5). They also provide an explanation for the persistence of radioactivity in the liver and other tissues of rats treated with [^{14}C]HCP.[3] Appreciable covalent binding of HCP metabolites to ether-extracted,

TCA-insoluble precipitates from the liver, kidney, and plasma was observed in rats treated either intraperitoneally or orally with [^{14}C]HCP (Table 6). The higher concentrations of covalently bound radioactivity in some tissues and organs may be associated with increased concentrations of reactive metabolites and/or a greater number of nucleophilic binding sites. With few exceptions, the distribution of covalently bound metabolites from HCP correlated well with the relative activities of microsomal cytochrome P-450 enzymes in the various tissues examined (23).

Two pathways may be involved in the covalent binding of HCP to microsomal protein through an arene oxide type of intermediate: (a) direct microsomal oxidation of the bisphenol to an arene oxide and (b) initial reductive dechlorination followed by arene oxide formation (Fig. 6). Chlorinated aromatic compounds, such as the isomeric tri- and tetrachlorobenzenes, are oxidized *in vivo* via an arene oxide type of intermediate (24). Since several metabolites of these polychlorinated benzenes are produced by an NIH shift (25) of a chlorine atom, the epoxide must be initially formed at a position already containing a halogen (24). Therefore it is possible that HCP could be metabolized directly to arene oxides by a similar pathway.

Arene oxide formation from HCP might also occur following an initial dechlorination reaction. A structurally related compound, hexachlorobenzene, can be converted *in vivo* to a number of metabolites reflecting the loss of 1, 2, or 3 chlorine atoms (26–29). A microsomal enzyme located in rat liver, lung, kidney, and intestine apparently catalyzed the reductive dechlorination of hexachlorobenzene (26). Although this microsomal system did not appear to require the addition of cofactors, one or more chlorophenols were formed from hexachlorobenzene when liver microsomal preparations were fortified with NADPH (26, 27). A similar NADPH- and oxygen-requiring microsomal system was

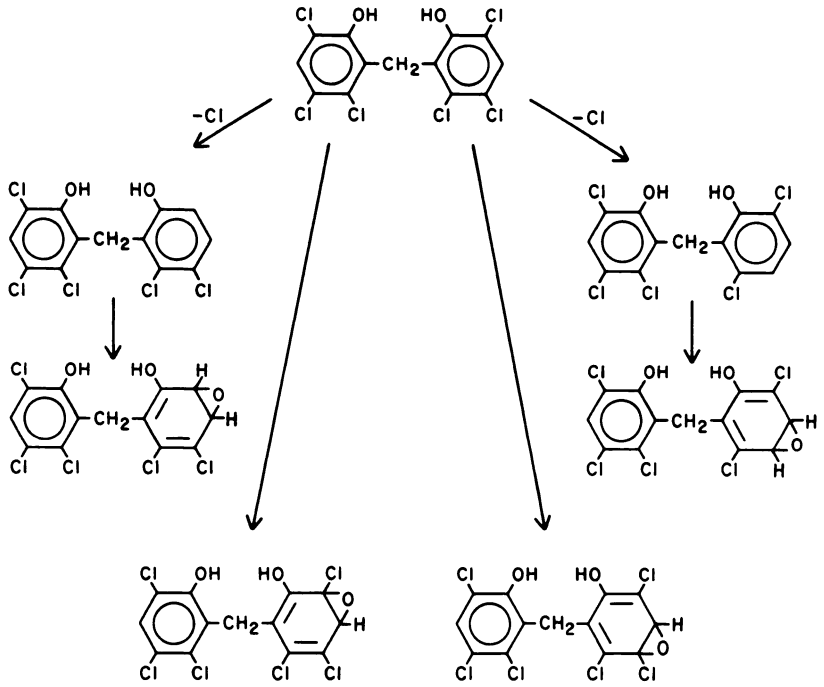

FIG. 6. *Possible routes of microsomal dechlorination and oxidation of hexachlorophene to reactive electrophiles*

described for the dechlorination of halogenated anesthetics (30). Reductive dechlorination of DDT analogues by liver preparations under anaerobic conditions was also demonstrated by Hassall and Manning (31), but the reaction may be catalyzed nonenzymatically by the reduced form of heme proteins (32).

At present we have no evidence that would indicate the relative importance of each pathway for the formation of arene oxides from HCP. However, the demonstration of several bound HCP metabolites by Raney nickel desulfurization and subsequent thin-layer chromatography suggested that both pathways may be functional *in vitro*.

The data presented are in agreement with the formation of reactive metabolites of HCP, presumably arene oxides, by cytochrome P-450-dependent monooxygenases in the endoplasmic reticulum. Subsequent covalent binding of the HCP epoxides or structurally related species to tissue proteins or other cellular macromolecules may result in an alteration in biochemical function that could play an important role in the expression of toxicity. HCP is a potent inhibitor of mixed-function oxidases and significantly reduces the content of microsomal cytochromes P-450 and b_5 at concentrations between 5 and 100 nmoles of HCP per milligram of microsomal protein (4). Since multiple forms of cytochrome P-450 hemoproteins in rat liver microsomes have been reported (33), the persistent binding of HCP to a microsomal protein with a molecular weight in the range 54,500–60,000 (Fig. 5) suggested that one of the binding sites may be cytochrome P-450. However, it should be emphasized that the effect of HCP on cytochrome P-450 may also represent a conversion to the inactive P-420 form as reported by Gandolfi *et al.* (4).

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