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EFFECT OF HEXACHLOROPHENE ON HEPATIC DRUG-METABOLIZING ENZYMES IN THE RAT*

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Abstract—Hexachlorophene (HCP) at oral doses of 30–50 mg/kg causes significant increases in hexobarbital sleeping time in Wistar and Sprague–Dawley rats, with the maximum effect occurring 6 hr after administration of the bisphenol. Liver microsomal *O*-demethylase activity is simultaneously reduced in rats receiving HCP. Incubation of rat liver microsomes with concentrations of HCP as low as 0·38 nmole/mg of microsomal protein *in vitro* inhibits the *O*-demethylase, nitroreductase and phenol UDP–glucuronyl transferase systems and also causes a reduction in the apparent content of cytochromes P-450 and b_5. The concentrations of HCP required to produce a 50 per cent inhibition or reduction in apparent cytochrome contents *in vitro* range between 4·7 and 98 nmoles HCP/mg of microsomal protein. Some evidence for a common inhibitory mechanism, perhaps involving interaction of HCP with the microsomal membrane, was obtained for the hepatic mixed function oxidase and cytochrome systems.

HEXACHLOROPHENE [2,2′-methylenebis-(3,5,6-trichlorophenol); HCP], a potent bactericide and fungicide, was widely used until recently in a variety of cosmetic and home care products. HCP has been shown to be toxic to rats by either oral or intraperitoneal administrations.[1–3] While spongy edema of the brain[2] and atrophy of the optic nerve[4] have been found in chronically exposed animals, HCP also produces hyperthermia in acutely intoxicated animals,[3] apparently as a result of uncoupling of oxidative phosphorylation.[5,6]

Many unexplained aspects of the toxicity of HCP together with the apparent resistance of HCP to oxidative metabolism and conjugation *in vitro*[7] prompted studies on the effect of HCP on drug metabolism *in vivo* and *in vitro*.

MATERIALS AND METHODS

Random bred adult male Wistar rats (200–400 g) from a closed colony in the Department of Agricultural Chemistry and male Sprague–Dawley rats (Horton Laboratories) of a similar size were used in this investigation. Rats were fasted overnight prior to treatment and then dosed at various times with corn oil solutions of HCP or 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (p,p′-DDT). The effect of HCP and p,p′-DDT (City Chemical Co., used as a positive control) on hexobarbital sleeping

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time was determined by the procedures of Dreyfuss *et al.*[8] after administration of 100 mg/kg of hexobarbital i.p. For determination of hepatic enzymatic activities, control animals and those treated with HCP or p,p'-DDT were sacrificed by cervical dislocation, and their livers removed rapidly and placed in cold 0·154 M isotonic KCl solution. Homogenates (10%, w/v) were prepared using a glass homogenizer tube with a Kel-F pestle. The homogenates were then centrifuged at 10,000 *g* for 20 min. The resulting supernatants were used for the analyses of enzymatic activities. Microsomes were isolated from untreated adult male Wistar rats to investigate the effect of HCP *in vitro* on hepatic microsomal drug metabolism. Microsomes were prepared at 0–4° from livers which were homogenized in 3 vol. of 0·154 M KCl, centrifuged at 10,000 *g* for 30 min, and the supernatant was centrifuged at 105,000 *g* for 1 hr. The microsomal pellet was finally suspended in 0·154 M KCl in such a manner that 1 ml suspension represented 1 g liver. Protein concentrations were determined by the procedure of Lowry *et al.*[9]

The method of Hoffman *et al.*[10] was utilized to measure *O*-demethylase activity using *p*-nitroanisole as substrate; nitroreductase activity was determined by the method of Hietbrink and DuBois[11] with *p*-nitrobenzoic acid as substrate; phenol UDP–glucuronyl transferase activity was measured by the procedure of Storey[12] with *p*-nitrophenol as acceptor. Incubation mixtures containing 2–6 mg microsomal protein in approximately 3 ml total volume were preincubated at 37° with HCP (2–3000 nmoles added in 50 μl acetone) for 5 min prior to the addition of substrate. Controls received 50 μl acetone alone. Nitroreductase and *O*-demethylase activities then were assayed after a 1-hr incubation period whereas a 20-min incubation period was employed for the glucuronyl transferase assays. The procedure of Ichikawa and Yamano[13] was used to determine the effects of HCP *in vitro* on the apparent concentration of cytochrome P-450. A similar spectrophotometric method was also employed to assess the effect of HCP on cytochrome b_5, which was assayed as described by Mazel.[14] For the cytochrome assays, the assay mixtures containing 4–6 mg microsomal protein in approximately 3 ml total volume (2 ml for the cytochrome b_5 assays) were preincubated at 37° for 10 min with HCP (20–6000 nmoles added in 10 μl acetone; controls contained 10 μl acetone alone), and the mixtures were then transferred to cuvets for assay of the cytochrome content. Glucose 6-phosphate dehydrogenase activity was measured spectrally at 340 nm for the production of NADPH. All assays of enzymatic activities were determined on a Gilford model 2000 spectrophotometer. Difference spectra for analysis of microsomal cytochromes were performed on a Cary model 15 spectrophotometer.

Data for the length of sleep and *O*-demethylase activities were analyzed for statistical difference by the *t*-test.[15]

Hexachlorophene, USP grade, was a gift from the Givaudan Corp. and was recrystallized (m.p. 165 to 165·5°) from isopropyl alcohol–water prior to use. Methylene-^{14}C-hexachlorophene (2·71 mCi/m-mole) was used in the binding and equilibrium dialysis studies.

RESULTS AND DISCUSSION

Pretreatment of two strains of rats with oral doses of HCP (50 mg/kg) in corn oil resulted in a prolonged hexobarbital sleeping time (Table 1). Significant increases in sleeping times were observed in Wistar rats 2 and 6 hr after dosing with HCP. The

TABLE 1. EFFECT OF HCP TREATMENT OF HEXOBARBITAL SLEEPING TIME IN TWO STRAINS OF RATS*

Strain and treatment	Dose (mg/kg)	Sleeping time† (min) 2 (hr)	6 (hr) Expt. 1	6 (hr) Expt. 2	12 (hr)
Wistar					
Control	0	87 ± 10	91 ± 9	88 ± 5	
HCP-treated	20			86 ± 32 (− 2·3%)	
HCP-treated	30			107 ± 12‡ (+ 21·6%)	
HCP-treated	40			141 ± 8‡ (+ 60·2%)	
HCP-treated	50	117 ± 11‡ (+ 24·7%)	153 ± 29‡ (+ 68·1%)		95 ± 42 (+ 7·4%)
p,p′-DDT-treated	50		51 ± 11‡ (− 43·9%)		
Sprague-Dawley					
Control	0	30 ± 9	22 ± 8		
HCP-treated	50	35 ± 5 (+ 16·7%)	39 ± 8§ (+ 43·5%)		

* From six to eight male rats received 100 mg/kg of hexobarbital i.p. at the indicated times after dosing with corn oil solutions of HCP p.o.

† Mean ± S.E. Values in parentheses are ± per cent of control animals that received corn oil alone.

‡ Highly significant at P ± 0·01.

§ Significant at P ± 0·05.

maximum effect occurred at 6 hr, the time when the highest concentrations of HCP and metabolites were known to be present in the liver after oral administration.* By 12 hr, sleeping times in HCP-treated rats had essentially returned to control values. Sleeping times were considerably shorter in the Sprague–Dawley rats; nevertheless, a significant increase in hexobarbital sleeping time was also found in this strain 6 hr after dosing with HCP. Treatment of Wistar rats with p,p′-DDT, a known inducer of microsomal enzymes, caused a 44 per cent reduction in the hexobarbital sleeping time 6 hr after administration of the pesticide.

The effect of HCP on sleeping times was related to the amount of HCP received by the rats (Table 1). The greatest response was observed with a HCP dose of 50 mg/kg, but sleeping times were then decreased in proportion to the HCP dose. When HCP pretreatment was reduced to 20 mg/kg, sleeping times were no longer affected.

In most instances, liver *O*-demethylase activity was also reduced in rats after dosing with 50 mg/kg of HCP p.o. (Table 2). In Wistar rats, the effect on the liver microsomal system was less pronounced than that on the hexobarbital sleeping time. A significant depression in *O*-demethylase values, however, was found in Sprague–Dawley animals 6 and 12 hr after pretreatment with HCP. These observations suggest that the increased hexobarbital sleeping time after administration of HCP results from a decreased rate of hexobarbital metabolism, either because of reduced

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TABLE 2. EFFECT OF HCP TREATMENT ON *O*-DEMETHYLASE ACTIVITY IN TWO STRAINS OF RATS*

Strain and treatment	*O*-demethylase activity† 1 (hr)	2 (hr)	6 (hr)	12 (hr)
Wistar				
Control	0·99 ± 0·11	0·93 ± 0·10	1·06 ± 0·14	1·11 ± 0·12
HCP-treated	0·98 ± 0·09 (− 1·0%)	0·89 ± 0·06 (− 3·2%)	0·92 ± 0·04 (− 13·2%)	1·02 ± 0·04 (− 8·1%)
Sprague–Dawley				
Control		1·28 ± 0·10	1·86 ± 0·39	2·37 ± 0·07
HCP-treated		1·45 ± 0·21 (+ 13·3%)	1·26 ± 0·17‡ (− 32·2%)	1·95 ± 0·05‡ (− 17·7%)

* Male rats received 50 mg/kg of HCP in corn oil p.o. at the indicated times prior to assay. Microsomes from two animals were used in each experiment and *O*-demethylase assays were carried out in triplicate.

† Activity in μg *p*-nitrophenol formed/hr/mg of microsomal protein. Mean ± S.E. reported. Values in parentheses are ± per cent of control animals that received corn oil alone.

‡ Highly significant at P ± 0·01.

levels of the hepatic drug-metabolizing enzymes (perhaps due to the elevated body temperatures of the dosed animals)[3] or from a direct inhibition of these enzymes by HCP.

The latter explanation seems more probable since HCP caused a marked decrease in the activities of several microsomal enzymes *in vitro* when liver microsomes from control rats were incubated with the bisphenol (Table 3). The *O*-demethylase and nitroreductase systems were inhibited at HCP concentrations of 0·38 and 1·2 nmoles/mg of microsomal protein respectively. Reduced microsomal enzyme activity did not result from impaired NADPH formation since glucose 6-phosphate dehydrogenase activity in the incubation mixtures was unaffected by HCP.

Hepatic UDP–glucuronyl transferase was also inhibited by HCP but to a lesser extent than the other microsomal enzyme systems (Table 3). Possible explanations for the relative insensitivity of the UDP–glucuronyl transferase system to inhibitors of microsomal NADPH–O_2-dependent mixed function oxidases have been previously reported.[16]

Incubation of HCP with rat liver microsomes also resulted in a decrease in the absorption of cytochromes P-450 and b_5 (Table 3). As cytochrome P-450 absorption decreased, a complementary peak appeared at 420 nm, apparently reflecting the conversion of cytochrome P-450 to P-420. Other chlorinated phenols[13] have also been shown to catalyze this conversion, probably elicited by an attack of the phenols on hydrophobic ligand bonds in the hemoprotein. Since HCP has surfactant or detergent properties at high concentrations[17] and can chelate metals,[18] it is noteworthy that detergents[19,20] and chelating agents[21] also have been shown to promote a conversion of P-450 to P-420. Non-ionic detergents can also inhibit reduction of microsomal cytochrome b_5, leading to lower levels of this cytochrome,[22] implying that other chemicals with surfactant properties, such as HCP, might have a similar effect.

TABLE 3. INHIBITION *in vitro* OF MICROSOMAL ENZYMATIC ACTIVITY AND DECREASE IN MICROSOMAL ABSORBANCY IN THE PRESENCE OF HEXACHLOROPHENE

HCP added (nmoles/mg microsomal protein)	Microsomal enzyme activity and apparent cytochrome content*				
	O-demethylase†	Nitroreductase‡	UDPGA transferase§	Cytochrome P-450‖	Cytochrome b_5¶
0	48·9 ± 5·7	7·6 ± 0·6	9·2 ± 0·6	1·08 ± 0·04	0·98 ± 0·0
0·38	42·4 ± 4·4 (14%)				
0·50			9·2 ± 0·5		
0·74	33·0 ± 4·5 (33%)				
1·0			9·3 ± 0·7 (10%)		
1·2		7·0 ± 0·2 (8%)			
1·7	34·5 ± 6·5 (29%)				
2·5	25·9 ± 2·9 (47%)				
4·9				1·02 ± 0·30 (5%)	0·85 ± 0·0 (13%)
5·0			8·0 ± 0·7 (13%)		
7·4	20·9 ± 5·8 (57%)				
9·9			7·8 ± 0·7 (15%)		
12		5·7 ± 0·8 (25%)			
37	9·4 ± 5·0 (81%)				
49		3·7 ± 0·5 (48%)	7·3 ± 0·5 (20%)	0·81 ± 0·07 (25%)	0·56 ± 0·03 (43%)
99			6·7 ± 0·4 (27%)		
120		2·1 ± 0·4 (73%)			
490				0·18 ± 0·10 (83%)	0·12 ± 0·07 (88%)
1240		0 (100%)			

* All enzyme activities reported as mean ± S.E. of approximately 4–6 determinations. Cytochrome contents are the mean of 2-3 determinations. Values in parentheses are per cent inhibition of enzyme activity or per cent decrease in apparent cytochrome content from controls. All experiments employed male Wistar rats.

† Values reported as nmoles *p*-nitrophenol formed/mg of microsomal protein/hr.

‡ Values reported as nmoles *p*-aminobenzoic acid formed/mg of microsomal protein/hr.

§ Values reported as nmoles *p*-nitrophenol consumed/mg of microsomal protein/20 min.

‖ Values reported as nmoles cytochrome P-450/mg of microsomal protein.

¶ Values reported as nmoles cytochrome b_5/mg of microsomal protein.

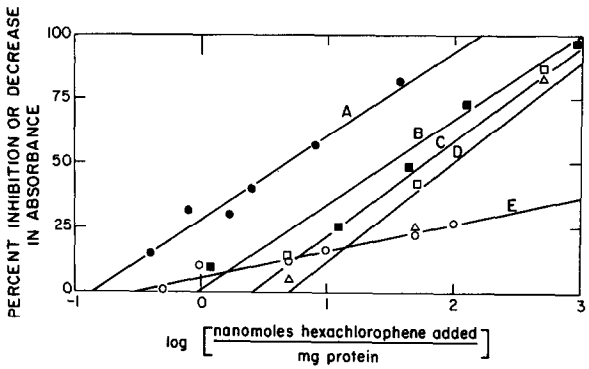

FIG. 1. Inhibition of microsomal enzymes and decrease in cytochromes P-450 and b_5 absorbancies by incubation *in vitro* with hexachlorophene: A (● = *p*-nitroanisole demethylase); B (■ = *p*-nitrobenzoic acid reductase); C (□ = decrease in apparent cytochrome b_5 concentration); D (△ = decrease in apparent cytochrome P-450 concentration); and E (○ = *p*-nitrophenol conjugation with glucuronic acid).

The relative influence of HCP on microsomal enzymes and cytochromes *in vitro* can be seen more clearly in a semilog plot of the data (Fig. 1). Under these conditions, 50 per cent inhibition of the *O*-demethylase and nitroreductase systems occurred at HCP concentrations of 4·7 and 32 nmoles/mg of microsomal protein respectively. A 50 per cent decrease in cytochromes P-450 and b_5 levels apparently occurred at somewhat higher HCP concentrations of 98 and 60 nmoles/mg of microsomal protein respectively. The cytochromes, however, were assayed after a shorter period of exposure than used in the enzymatic assays. A longer exposure to HCP might, therefore, result in substantially reduced cytochrome levels.

With the exception of glucuronyl transferase, inhibition of the other microsomal systems examined followed parallel per cent inhibition–HCP concentration plots, implying a common inhibitory mechanism. Low concentrations of HCP also affect various other cellular or subcellular membrane-associated systems, causing inhibition of oxidative phosphorylation in rat liver mitochondria,[6] leakage of cellular constituents from bacteria[17] and altering cation transport in erythrocytes.* These observations suggest the possibility that the inhibition of various microsomal enzyme systems might also occur as a result of a similar interaction of HCP with microsome-associated systems.

In support of such a mechanism, HCP was found to bind strongly to rat liver microsomes. When a typical microsomal reaction mixture was incubated with 100 nmoles of ^{14}C-labeled HCP/mg of microsomal protein for 1 hr, over 89 per cent of the radioactivity remained bound to recovered and washed microsomes. Equilibrium dialysis experiments showed that microsomes could bind a maximum of 307 nmoles HCP/mg of microsomal protein. Such strong association of HCP with microsomes probably reflected either hydrophobic binding between the hydrophobic moiety of HCP to the membrane or hydrogen bonding to the peptide linkage[23] of the microsomal proteins.

The concentration of HCP required to inhibit hepatic mixed function oxidases is comparable to that reported for other inhibitors of these systems.[24] Moreover, inhi-

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bition of the microsomal UDP–glucuronyl transferase system by HCP occurs at concentrations similar to those found for other phenolic inhibitors of glucuronide formation.[12] Whether the effects of HCP on hepatic enzymes *in vivo* and *in vitro* and the mechanism for HCP inhibition of these hepatic systems are similar remains to be elucidated.

These results suggest the possibility that a continual exposure of individuals to HCP through the use of various products containing this antibacterial agent or the continued use of such products after HCP has been replaced by other germicidal agents could alter the individual's sensitivity to other drugs or chemicals because of the presence or sudden absence of a HCP-inhibitory effect on liver microsomal systems.

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