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EFFECT OF HEXACHLOROBENZENE ON MICROSOMAL ENZYME SYSTEMS

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Abstract—Male rats were fed for 10 days on a diet containing 333 ppm hexachlorobenzene. Increased microsomal protein levels were noted compared to control rats. On a per g liver basis, the levels of aniline hydroxylase, biphenyl 4-hydroxylase, biphenyl 2-hydroxylase, 4-nitroanisole *O*-demethylase, esterase, cytochrome P-450 and cytochrome b_5 all increased compared with the control values. On a per mg microsomal protein basis, biphenyl 2-hydroxylase, 4-nitroanisole *O*-demethylase and cytochrome P-450 levels increased several-fold compared with the control values. It is suggested that, by inducing the 2-hydroxylation reaction, hexachlorobenzene might cause preferential *ortho*-hydroxylation, as do some carcinogenic polycyclic hydrocarbons, and that in some circumstances this could lead to the formation of carcinogens.

HEXACHLOROBENZENE (HCB) is a fungicide that has been used extensively as a seed dressing for the control of bunt fungi (*Tilletia* spp) in cereal crops. HCB has been shown to be associated with the production of porphyria in human beings and experimental animals[1–3] and is known to induce increased activity of mitochondrial delta-aminolaevulinic acid synthetase.[4] Wada *et al.*[5] showed that mice treated with various porphyria-inducing chemicals, including HCB, had increased levels of cytochrome P-450 in the liver. Storage of dieldrin in rats was depressed by HCB in the diet[6] and a protective effect against ryegrass staggers[7,8] was noticed in sheep dosed with HCB.*

The present investigation was undertaken to study the effect of a high level of dietary HCB on several microsomal enzyme systems.

MATERIALS AND METHODS

Source of materials. NADP, glucose 6-phosphate monosodium salt, 2-hydroxy- and 4-hydroxybiphenyl and bovine serum albumin were obtained from Sigma Chemical Co., U.S.A. Biphenyl and HCB (micro-analytical grade) were supplied by B.D.H., Poole, England.

The aniline was redistilled with zinc dust before use. To remove any free phenol, phenyl acetate was redistilled and passed through a basic alumina column using petroleum ether as the eluting solvent. 4-Nitroanisole was recrystallized from water.

Preparation of animals. Twelve 5-week-old male rats of the Dunedin–Wistar strain

* M. Avrahami, personal communication.

maintained on diet 86 (shown to have < 0·05 ppm HCB, DDT and DDE)* were divided into two groups of six. One group served as controls and the other group were fed for 10 days with 333 ppm HCB in the standard rat diet.

Preparation of liver samples. All rats were starved for 12 hr and killed by cervical dislocation. The livers were removed immediately, blotted dry and weighed. All enzyme studies were conducted using either 12,000 *g* supernatants or microsomal preparations. Livers were homogenized in 4 vol. (w/v) ice-cold 1·15% KCl, pH 7·4, in a glass homogenizer with a Teflon pestle and centrifuged for 15 min at 12,000 *g* at 4°. The pellet was discarded and the supernatant filtered through a small plug of cotton–wool. The 12,000 *g* supernatant was diluted with ice-cold 1·15% KCl, pH 7·4, so that the 2·0 ml supernatant was equivalent to that obtained from 1 g liver. This supernatant was used for the enzyme assays. A microsomal pellet was prepared by centrifuging a portion of the diluted supernatant at 77,000 *g* for 80 min at 4°. The sedimented microsomes were resuspended in 1·15% KCl, pH 7·4, and recentrifuged. The washed pellet was resuspended in 1·15% KCl, pH 7·4 and used immediately for protein, cytochrome P-450 and cytochrome b_5 assays.

Assays. Cytochrome P-450 was determined by measuring the carbon monoxide-difference spectrum of a sodium dithionite-reduced microsomal suspension in 0·05 M phosphate buffer, pH 7·4. The molar extinction coefficient was assumed to be 91 cm^{-1} mM^{-1}.[9]

Cytochrome b_5 was determined by measuring the difference spectrum of a sodium dithionite-reduced microsomal suspension in 0·05 M phosphate buffer, pH 7·4. The molar extinction coefficient was assumed to be 171 cm^{-1} mM^{-1}.[9]

Microsomal protein estimations were made by the biuret method[10] using bovine serum albumin as a standard.

Esterase activity was assayed by a continuous spectrophotometric method based on the method of Zeller.[11] The rate of phenol formation from the hydrolysis of phenyl acetate in 0·05 M phosphate buffer, pH 7·4, was recorded at 270 nm and 37° in a thermostatted cuvette holder.

Aniline hydroxylase activity was measured by the quantitation of the 4-amino-phenol formed, and was based on the method of Brodie and Axelrod.[12]

4-Nitroanisole *O*-demethylase activity was measured continuously at 37° in a recording spectrophotometer fitted with a thermostatted curvette holder. The method was based on that described by Zannoni.[13]

Biphenyl hydroxylation was assayed by measuring the formation of 4-hydroxybiphenyl and 2-hydroxybiphenyl. The metabolites were extracted and quantitated by the fluorimetric method of Creaven *et al.*[14] The excitation and emission wavelengths used were ex. 265 nm, em. 350 nm and ex. 286 nm, em. 425 nm. These figures are uncorrected instrumental values (Baird atomic fluorispec).

All the microsomal oxidation reactions utilizing the 12,000 *g* supernatant contained the following final concentrations in the incubation system: NADP, 0·24 mM; glucose 6-phosphate, 6 mM; $MgCl_2$, 5 mM. The assay systems did not require nicotinamide. The final concentrations of the substrates used were as follows: 2 mM, aniline; 1·1 mM, biphenyl; and 1·2 mM 4-nitroanisole. The final volume of the incubation mixture for the hydroxylase assays was 6 ml, and the flasks were shaken at 100 oscillations/min at 37° for 15 min.

* DDT, dichloro diphenyl trichloroethane; DDE, dichloro diphenyl dichloroethylene.

RESULTS AND DISCUSSION

The results are summarized in Table 1, from which it is evident that hepatic microsomal protein levels increased almost two-fold. On a per g liver basis, all the enzyme activities that were measured increased. The increase in levels of aniline hydroxylase, esterase and biphenyl 4-hydroxylase in the dosed animals is in a 1:1 proportion with the increase in microsomal protein that occurs. The amount of cytochrome b_5 increases similarly. These values reflect the increase in protein synthesis that occurs in the treated animals. There is a much greater increase in the amount of cytochrome P-450, indicating a specific synthesis of this component. This induction phenomenon with certain drugs and pesticides has been well documented in recent times,[15–17] although few reports have been published on the induction of esterases. Remmer[18] found increased hydrolysis of procaine in rabbits dosed with phenobarbital, and Benöhr *et al.*[19] showed increased microsomal esterase activity in rats and rabbits after dosing with phenobarbital, although no increase in specific activity was observed. These esterases are NADPH-independent and not part of the microsomal oxidation system, although Douch *et al.*[20] have reported an NADPH-dependent "hydrolytic" cleavage of carbamates.

The increases in the rate of *O*-demethylation of 4-nitroanisole and the 2-hydroxylation of biphenyl, after dosing, were greater than expected based simply on an increase in total liver weight or total cytochrome P-450. Both 4-nitroanisole and biphenyl are classed as type I substrates, and it may be that the ease of binding of this type of substrate–P-450 interaction increases after induction with HCB due, perhaps, to an allosteric effect caused by the inducing agent or its metabolites. According to Mannering,[21] responses by cytochrome P-450 to some drugs might be due to differences in the environment of the haemoprotein which may determine the degree of binding of drugs to type I and type II binding sites.

The large increase in biphenyl 2-hydroxylase activity expressed either per g liver or per mg microsomal protein is of particular interest. Biphenyl is largely metabolised to 4-hydroxybiphenyl, but in some species, particularly young animals, 2-hyd-

TABLE 1. EFFECT OF HEXACHLOROBENZENE ON SOME HEPATIC MICROSOMAL PARAMETERS OF MALE RATS*

Parameter	Control (per mg microsomal protein)	Dosed (per mg microsomal protein)	Control (per g liver)	Dosed (per g liver)
Protein†			15·6 ± 1·2	28·2 ± 1·4‡
Aniline hydroxylases§	0·72 ± 0·05	0·70 ± 0·04	10·9 ± 0·4	19·7 ± 1·2‡
4-Nitroanisole *O*-demethylase‖	3·8 ± 0·4	9·8 ± 0·5‡	57·1 ± 2·3	280·2 ± 22·4‡
Biphenyl 4-hydroxylase¶	3·26 ± 0·28	4·13 ± 0·18**	49·5 ± 1·8	115·4 ± 3·2‡
Biphenyl 2-hydroxylase††	0·06 ± 0·00	0·22 ± 0·02‡	0·82 ± 0·07	6·10 ± 0·35‡
Esterase‡‡	1·68 ± 0·08	1·66 ± 0·04	26·3 ± 2·2	46·6 ± 2·3‡
Cytochrome b_5§§	0·28 ± 0·03	0·30 ± 0·06		
Cytochrome P-450‖‖	1·05 ± 0·09	1·82 ± 0·07‡		

* Values represent mean of six animals ± S.E.M.
† Mg microsomal protein.
‡ Value significantly different from control (P = 0·001).
§ Nmoles 4-aminophenol formed/min.
‖ Nmoles 4-nitrophenol formed/min.
¶ Nmoles 4-hydroxybiphenyl formed/min.
** Value significantly different from control (P = 0·05).
†† Nmoles 2-hydroxybiphenyl formed/min.
‡‡ Micromoles phenol formed/min.
§§ Nmoles cytochrome b_5.
‖‖ Nmoles cytochrome P-450.

roxybiphenyl is formed.[14] Creaven *et al.*[14] suggested that both types of hydroxylation were effected by different microsomal enzyme systems. Using compounds known to induce microsomal enzymes, Creaven and Parke[22] showed that phenobarbitone, nikethamide and meprobamate stimulate predominantly the 4-hydroxylation of biphenyl in both the rat and the mouse. However, the carcinogenic polycyclic hydrocarbons, 3,4-benzpyrene, 20-methylcholanthrene and 1,2,5,6-dibenzanthracene preferentially induced the 2-hydroxylation of biphenyl whereas non-carcinogenic polycyclic hydrocarbons did not. The induction of the 2-hydroxylation may cause preferential formation of *ortho*-hydroxylated metabolites from suitable aromatic compounds. In the case of some polycyclic amines, this would increase the production of *ortho*-aminophenol metabolites which are active carcinogens. As our results show that HCB also preferentially stimulates the 2-hydroxylation, it is interesting to speculate that HCB might be a potential hazard, apart from its known porphyria-inducing effect, in causing enzyme induction and preferential *ortho*-hydroxylation and thus with suitable molecules forming potential carcinogens.

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