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EFFECT OF HEXACHLOROBENZENE ON HEPATIC MICROSOMAL ENZYMES IN THE RAT

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Abstract—Short term feeding of the fungicide, hexachlorobenzene, to rats causes a marked increase in hepatic cytochrome P-450 levels, enhancement of the activities of several *in vitro* microsomal oxidations, diminished pharmacological actions of hexobarbitone and zoxazolamine *in vivo*. Chronic feeding of rats with hexachlorobenzene leads to a diminished pharmacological action *in vivo* of zoxazolamine but not hexobarbitone in male rats; in contrast to the male rat, there is a diminished pharmacological response to hexobarbitone *in vivo* in the female rat. The concentration of cytochrome P-450 in the liver remains elevated throughout the chronic feeding of hexachlorobenzene. The findings suggest that hexachlorobenzene is an inducer of the phenobarbitone class but there is a qualitative difference between phenobarbitone and hexachlorobenzene with regard to microsomal enzyme induction.

THE DURATION of action of many drugs in the whole animal is determined by the activity of the hepatic microsomal enzymes which inactivate these drugs. The activity of the hepatic microsomal drug-metabolizing system can be enhanced or depressed by the administration or feeding of a wide variety of compounds to animals.[1] Most compounds which enhance the activity of this system are themselves substrates for the system and are therefore inactivated by it.

Chronic feeding of the fungicide hexachlorobenzene (HCB), a chemically and biologically stable molecule,[2] to the rat leads to an hepatic porphyria.[3,4] Prior to the development of this porphyria, HCB causes changes in the activity of the hepatic drug-metabolizing system *in vitro* and in the response of certain drugs administered *in vivo*. These changes which have already been presented in preliminary form[5] are reported in this study.

MATERIALS AND METHODS

All rats used in these studies were of the Porton strain. For the chronic feeding experiments, rats (90–110 g initial body wt) were fed their M.R.C. 41B diet in powdered form or the same containing 0·2% w/w hexachlorobenzene *ad lib*. For the short term feeding experiments, rats of the following ages were fed their diet in powdered form or the same containing 0·2% w/w hexachlorobenzene *ad lib*. for 7 days,

Male immature	(initial age 25–30 days)
male mature	(initial age 65–70 days)
Female	(initial age 25–30 days)

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Determination of pharmacological action of drugs. The duration of the pharmacological actions of hexobarbitone sodium* (150 mg/kg body wt) and zoxazolamine† (100 mg/kg body wt) was compared in rats fed hexachlorobenzene in the diet and control rats by measuring respectively the "sleeping time" and "paralysis time" following i.p. injections of the drugs. The "sleeping time" and "paralysis time" were taken as the time elapsing between injection of the drug and the recovery of the righting reflex.

Plasma hexobarbitone. Hexobarbitone was assayed by the method of Brodie *et al.*[6] as modified by Cooper and Brodie.[7]

Plasma zoxazolamine. Zoxazolamine was assayed according to the method of Burns *et al.*[8]

Isolation of hepatic microsomes. Homogenates of rat liver (10% w/v) were prepared in 250 mM sucrose. The nuclei and mitochondria were sedimented by centrifugation of the homogenate at 9000 *g* for 20 min. The microsomes, which were obtained by centrifugation of the resulting supernatant fraction at 100,000 *g* for 60 min, were washed once in 1·15% KCl and finally suspended in 100 mM phosphate buffer pH 7·4 containing 1 mM EDTA so that 1 ml contained the microsomes from approximately 0·3 g liver.

Enzyme assays

Hexobarbitone oxidase. The reaction mixture (3 ml) contained sodium-potassium phosphate buffer pH 7·4 (167 μmoles), nicotinamide (50 μmoles), glucose-6-phosphate (12 μmoles), magnesium chloride (6·7 μmoles), NADP (0·16 μmole), glucose-6-phosphate dehydrogenase (0·2 unit), hexobarbitone sodium (1·6 μmoles) and microsomal protein, 1 mg/ml. The oxidation of hexobarbitone was determined after 15 min incubation at 37° by the disappearance of the substrate as already described.[6,7]

Zoxazolamine hydroxylase. The metabolism of zoxazolamine by hepatic microsomes was performed in the medium described for hexobarbitone oxidase except that zoxazolamine (0·59 μmole) replaced hexobarbitone. The hydroxylation of zoxazolamine was determined after 20 min incubation at 37° by the disappearance of the substrate according to Conney *et al.*[9]

All assays were performed in air with constant shaking.

Estimation of cytochrome P-450. The content of microsomal P-450 was determined spectrophotometrically according to Omura and Sato[10] using an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in extinction at 450 and 490 nm.

Protein estimation. Protein was estimated by the biuret reaction according to Robinson and Hogden[11] as modified by Aldridge.[12]

Special reagents. Hexachlorobenzene (organic analytical reagent grade) was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Hexobarbitone sodium was kindly donated by May & Baker Ltd., Dagenham, Essex, U.K. and zoxazolamine was a gift from McNeil Laboratories Inc., Fort Washington, Pennsylvania, U.S.A.

RESULTS AND DISCUSSION

Long term experiments. Chronic feeding of HCB to rats leads to an increase in liver weight and microsomal protein.[13] Concomitant with these changes there is an increase in the concentration of the microsomal haemoprotein, cytochrome P-450 in

* Sodium 5-(1-cyclohexen-1-yl)-1,5-dimethylbarbiturate.

† 2-Amino-5-chlorobenzoxazole.

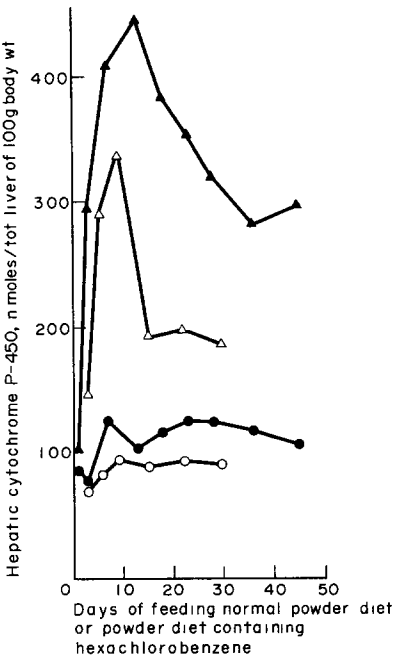

FIG 1. Effect of long term feeding of hexachlorobenzene on hepatic cytochrome P-450 level of rats. (●) Male control; (▲) male hexachlorobenzene; (○) female control; (△) female hexachlorobenzene. Each point represents the mean value for at least two rats and not more than three rats.

the livers of rats fed HCB (Fig. 1); a peak value is found between 7–13 days of feeding HCB, after which the concentration declines to a steady value but remains 2–3 fold above that of control. It should be emphasized at this point that hepatic porphyrins only accumulate in the liver after about 70 days of feeding HCB.[14]

These changes in hepatic cytochrome P-450 concentration are accompanied by changes in the pharmacological response to certain drugs. In male rats HCB decreases the "sleeping time" following administration of hexobarbitone (Fig. 2); this decrease is seen only during the initial three weeks of feeding HCB when the hexobarbitone "sleeping time" in normal male rats is age-dependent, as originally shown by Quinn *et al.*[15] When the "sleeping time" in normal male rats becomes steady at around 7 weeks of age, HCB does not cause any decrease in the "sleeping time". In contrast to the male rat, the hexobarbitone "sleeping time" in normal female rats is longer but is not age-dependent; HCB decreases the "sleeping time" by approximately 50 per cent at all intervals studied (Fig. 3).

Although the duration of action of hexobarbitone in the rat is sex-dependent, the "paralysis time" following zoxazolamine administration is not.[16] Chronic feeding of HCB to the male rat causes a marked decrease in the "paralysis time" at all intervals studied (Fig. 4).

A comparison of Fig. 1 with Figs. 2–4 indicates that the levels of cytochrome P-450 are not directly related to the changes in the duration of action of the two drugs brought about by HCB; furthermore, the adult male rat (after about 50 days of feeding HCB) did not show a decreased hexobarbitone "sleeping time" (as compared with

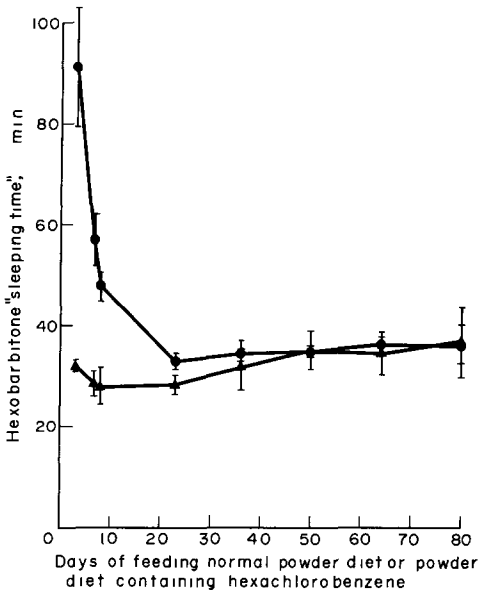

FIG. 2. Effect of long term feeding of hexachlorobenzene on hexobarbitone "sleeping time" in male rats. (●) Control; (▲) hexachlorobenzene. Each point represents the mean value ± S.E.M. for at least three rats and not more than six rats.

the corresponding controls) despite an increased hepatic cytochrome P-450 level. Two possibilities may explain the lack of stimulation by HCB of the metabolism of hexobarbitone. Either HCB may initially stimulate and then inactivate the hexobarbitone metabolizing system so that after 50 days of feeding HCB, no further stimulation is apparent or the failure of HCB to stimulate the metabolism of hexobarbitone *in vivo* may depend on the "unresponsiveness" of the adult male rat (relative

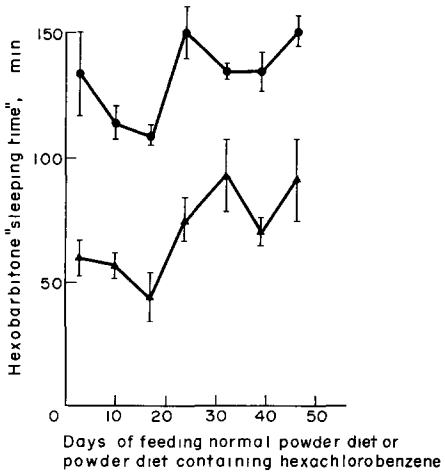

FIG. 3. Effect of long term feeding of hexachlorobenzene on hexobarbitone "sleeping time" in female rats. (●) Control; (▲) hexachlorobenzene. Each point represents the mean value ± S.E.M. for at least three rats and not more than five rats.

to the immature rat) to induction by a foreign compound of this metabolic pathway.[1] In order to distinguish between these possibilities and because the duration and intensity of action of drugs such as hexobarbitone,[17,18] zoxazolamine[17] carisoprodol[19] and strychnine[18] are largely regulated by the levels of the hepatic microsomal drug-metabolizing system in which cytochrome P-450 participates, a study has been undertaken to determine whether a direct correlation exists between *in vivo* duration of action of hexobarbitone and zoxazolamine and *in vitro* metabolism of these drugs after short term feeding of HCB. Also, because changes in "sleeping" and "paralysis" times elicited by chronic feeding of HCB have resembled the response to phenobarbitone, certain comparisons between HCB and phenobarbitone have been made in this study. Furthermore, these comparisons have been made between (a) immature and adult male rats and (b) male and female rats of the same age after seven days of feeding HCB or two days phenobarbitone pretreatment.

TABLE 1. EFFECT OF SHORT TERM ADMINISTRATION OF HEXACHLOROBENZENE AND PHENOBARBITONE ON DURATION OF ACTION OF HEXOBARBITONE AND PLASMA THRESHOLD LEVEL OF HEXOBARBITONE IN RATS AND HEXOBARBITONE OXIDASE ACTIVITY OF RAT HEPATIC MICROSOMES

	Duration of action of hexobarbitone (min)		
	Control	Hexachlorobenzene	Phenobarbitone
Male immature	64·3 ± 3·8 (15)	32·0 ± 2·6* (3)	24·0 ± 3·5* (3)
Male mature	35·0 ± 1·9 (19)	27·4 ± 1·2* (8)	28·7 ± 1·9 (11)
Female	147·8 ± 6·0 (22)	44·7 ± 2·9* (13)	19·6 ± 0·2* (8)
	Plasma level of hexobarbitone on recovery (μg/ml plasma)		
Male immature	62·6 ± 3·7 (16)	61·2 ± 9·6 (7)	79·4 ± 3·7* (7)
Male mature	67·0 ± 2·8 (13)	59·8 ± 1·4 (5)	78·7 ± 1·6* (8)
Female	79·8 ± 5·1 (17)	71·8 ± 2·8 (5)	109·8 ± 8·7* (7)
	Hepatic microsomal activity (nmoles hexobarbitone metabolized/min/mg mic. protein)		
Male immature	1·65 ± 0·21 (13)	4·50 ± 0·73* (6)	3·42 ± 0·13* (5)
Male mature	3·00 ± 0·36 (9)	5·25 ± 0·63* (6)	3·50 ± 0·10 (4)
Female	1·08 ± 0·20 (5)	3·21 ± 0·55* (6)	4·31 ± 0·45* (4)

Rats were fed normal powder diet or the same containing 0·2% w/w hexachlorobenzene for 7 days or were given phenobarbitone sodium (80 mg/kg) 16 hr and 40 hr prior to killing. Results are expressed as mean ± S.E.M. with number of observations in parentheses.

* $P < 0·01$ with respect to controls.

Short term experiments. Both HCB and phenobarbitone produce a decrease in the hexobarbitone "sleeping time" and stimulate the hepatic metabolism of hexobarbitone *in vitro* (Table 1). Although seven days of feeding HCB to adult male rats caused a small but significant decrease in the hexobarbitone "sleeping time" (Table 1) and chronic feeding of HCB for 50 days did not (Fig. 2) it still seems probable that the major reason why HCB does not decrease the hexobarbitone "sleeping time" during chronic feeding is that the hexobarbitone metabolizing system in the adult male rat becomes less and less responsive to an inducer such as HCB. Unfortunately, no studies on *in vitro* hepatic metabolism of hexobarbitone were performed to confirm this latter point. In the female rat, the hexobarbitone "sleeping time" is decreased more markedly after 7 days of feeding HCB (Table 1) than after chronic feeding for 45 days (Fig. 3) indicating a slight loss of response to HCB induction or alternatively

TABLE 2. EFFECT OF SHORT TERM FEEDING OF HEXACHLOROBENZENE ON DURATION OF ACTION OF ZOXAZOLAMINE IN RATS, ZOXAZOLAMINE HYDROXYLASE ACTIVITY OF RAT HEPATIC MICROSOMES AND MICROSOMAL PROTEIN

	Duration of action of zoxazolamine (min)	
	Control	Hexachlorobenzene
Male immature	171·6 ± 14·4 (11)	17·5 ± 2·5* (4)
Male mature	304·5 ± 16·1 (8)	60·1 ± 3·2* (9)
Female	141·1 ± 8·7 (8)	18·2 ± 1·9* (6)
	Hepatic microsomal activity (nmoles zoxazolamine metabolized/min/mg mic. protein)	
Male immature	0·27 ± 0·02 (4)	1·38 ± 0·03* (4)
Male mature	0·22 ± 0·04 (4)	0·74 ± 0·12* (4)
Female	0·33 ± 0·05 (7)	1·35 ± 0·11* (8)
	Microsomal protein. (mg/g wet wt liver)	
Male immature	16·7 ± 0·6 (22)	20·8 ± 0·4* (14)
Male mature	18·1 ± 0·8 (15)	17·4 ± 0·7 (4)
Female	16·5 ± 0·8 (14)	19·9 ± 0·4* (10)

Rats were fed normal powder diet or the same containing 0·2% w/w hexachlorobenzene for 7 days.

Results are expressed as mean ± S.E.M. with number of observations in parentheses.

* $P < 0.01$ with respect to controls.

a slight inhibition of the hexobarbitone metabolizing system. In contrast, the zoxazolamine "paralysis time" is markedly decreased and the *in vitro* metabolism increased after seven days of feeding HCB (Table 2) and the "paralysis time" is still decreased markedly after 50 days of HCB feeding (Fig. 4).

A comparison between HCB and phenobarbitone in this study has shown that HCB behaves qualitatively as an inducer of the phenobarbitone class.[17] The stimu-

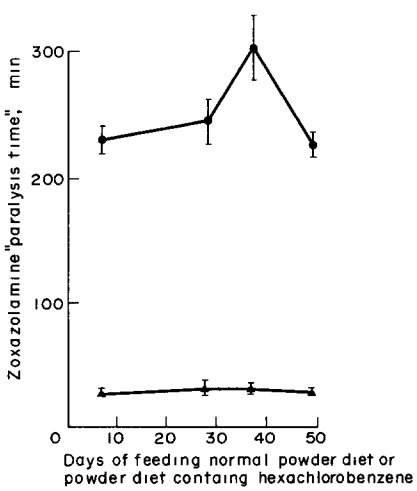

FIG. 4. Effect of long term feeding of hexachlorobenzene on zoxazolamine "paralysis time" in male rats. (●) Control; (▲) hexachlorobenzene. Each point represents the mean value ± S.E.M. for at least three rats and not more than four rats.

lation of the metabolism of hexobarbitone (Table 1) and zoxazolamine (Table 2) and also the *N*-demethylation of aminopyrine and the *p*-hydroxylation of aniline (unpublished observations) shows that HCB resembles the class of inducers typified by phenobarbitone, rather than 3-methylcholanthrene.

In order to see whether a direct correlation exists between the duration of action of hexobarbitone and zoxazolamine *in vivo* and hepatic metabolism of these drugs *in vitro*, the hexobarbitone "sleeping times" and zoxazolamine "paralysis times" have been plotted as reciprocals against their respective hepatic metabolism *in vitro* (Figs. 5 and 6). Figure 5 shows that if a best straight line is drawn through the points derived from control and phenobarbitone data, an inverse correlation does exist between the duration of action of hexobarbitone *in vivo* and the hepatic metabolism of hexobarbitone *in vitro*. However, the points derived from HCB data clearly fall below the line. It is clear that the duration of the pharmacological response is longer than might be expected from the stimulation of metabolism of hexobarbitone *in vitro* in HCB-fed rats. This need not necessarily imply that a different kind of correlation exists between *in vivo* response to hexobarbitone and *in vitro* hepatic metabolism according to the two inducers but that HCB, in some way, intensifies the pharmacological response of the rat to hexobarbitone e.g. by a change in the sensitivity of the hexobarbitone drug receptor or by a redistribution of hexobarbitone between the plasma and extravascular tissues. Figure 6 shows that there is a correlation between the duration of action of zoxazolamine *in vivo* and hepatic metabolism of zoxazolamine *in vitro* for data derived from control and HCB-treated rats. However, it should be pointed out that the *in vitro* zoxazolamine metabolism data derived from the liver microsomes of HCB-fed rats is an underestimate since the rate of metabolism was

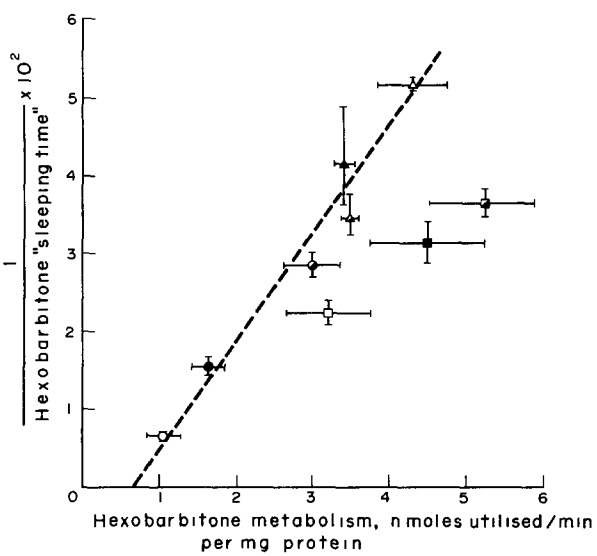

FIG. 5. The relationship between the duration of action of hexobarbitone *in vivo* and hepatic metabolism of hexobarbitone *in vitro*. All the data in this figure have been derived from short term experimental results contained in Table 1 and have been plotted as mean ± S.E.M. in both directions. (●) Control immature male; (■) hexachlorobenzene immature male; (▲) phenobarbitone immature male; (◑) control mature male; (◪) hexachlorobenzene mature male; (◭) phenobarbitone mature male; (○) control female; (□) hexachlorobenzene female; (△) phenobarbitone female.

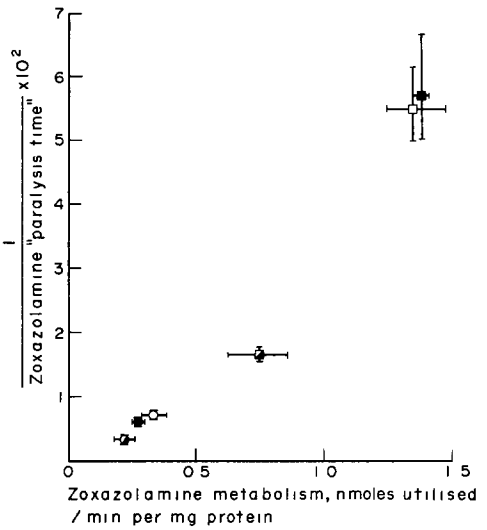

FIG. 6. The relationship between the duration of action of zoxazolamine *in vivo* and hepatic metabolism of zoxazolamine *in vitro*. All the data in this figure have been derived from short term experimental results contained in Table 2 and have been plotted as mean ± S.E.M. in both directions. (●) Control immature male; (■) hexachlorobenzene immature male; (◑) control mature male; (◪) hexachlorobenzene mature male; (○) control female; (□) hexachlorobenzene female.

not linear for 20 min as in the control microsomes. In the absence of zoxazolamine data derived from phenobarbitone-treated rats, it is not possible to say whether there are differences between HCB and phenobarbitone with respect to zoxazolamine metabolism.

Because of the possible changes in the pharmacological response to hexobarbitone in HCB-fed rats, measurement has been made of the plasma levels of hexobarbitone at recovery of the righting reflex. A comparison of the plasma levels of hexobarbitone in control and HCB-treated rats shows that HCB-treated rats recover their righting reflex at plasma hexobarbitone levels not significantly lower than the corresponding controls. Rats pretreated with phenobarbitone have plasma levels of "hexobarbitone" raised above corresponding controls (Table 1). The reason for these increases is that the method of assay is not specific for hexobarbitone but also measures residual plasma phenobarbitone. However, if the plasma level measured in phenobarbitone treated rats (not given hexobarbitone) is subtracted from the plasma level measured in phenobarbitone treated rats (given hexobarbitone) then the plasma level of hexobarbitone can be obtained. Such a calculation has been made for phenobarbitone–pretreated female rats ($109{\cdot}8 \pm 8{\cdot}7 - 39{\cdot}1 \pm 3{\cdot}9 = 70{\cdot}7 \pm 9{\cdot}5$ which is not significantly different from the control level of $79{\cdot}8 \pm 5{\cdot}1$ μg/ml plasma). Moreover, this calculation and the observation that rats pretreated with phenobarbitone and then given an anaesthetic dose of hexobarbitone recover their righting reflexes at a plasma barbiturate level higher than the corresponding control infers that the plasma phenobarbitone contribution is pharmacologically inert as compared to the hexobarbitone.

These observations of the influence of hexachlorobenzene upon hepatic microsomal activity form part of a study of the mechanism by which HCB produces an hepa-

tic porphyria. Observations relating to the production of hepatic porphyria by HCB are to be reported elsewhere.[14]

Finally, short term studies on hepatic enzyme induction by polychlorinated biphenyls[20] (PCB's) and long term studies on the porphyrogenic action of PCB's[21] suggest that these compounds and HCB have several actions in common. Studies are currently in progress to confirm whether or not such similarities in action do exist.

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