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MIXED TYPE HEPATIC MICROSOMAL ENZYME INDUCTION BY HEXACHLOROBENZENE

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Abstract—A comparison of microsomal spectral changes induced by 3-methylcholanthrene, phenobarbitone and hexachlorobenzene has been recorded. In addition, changes after hexachlorobenzene treatment of microsomal haem turnover in 'CO-binding particles' have been made. Hexachlorobenzene, like 3-methylcholanthrene, alters the spectral characteristics of cytochrome P-450 by shifting its peak to 448 nm. Administration of 5-amino[4-^{14}C]laevulinate to untreated and hexachlorobenzene-treated rats leads to the incorporation of radioactivity into the haemoprotein(s) of the CO-binding particles obtained from liver microsomes. There is a biphasic loss of radioactivity from the CO-binding particles in both normal and hexachlorobenzene-treated rats. Half-life determinations show that hexachlorobenzene increases the half-life of the fast phase component but not the slow phase component. As a result of these and previous studies, it is proposed that hexachlorobenzene is representative of a new class of inducers which shares the properties of both the polycyclic hydrocarbon group and the phenobarbitone, chlordane group.

Feeding of hexachlorobenzene (HCB) to rats causes an increase in the amount of hepatic microsomal haemoprotein, cytochrome P-450, enhancement of the microsomal oxidations of hexobarbitone and zoxazolamine *in vitro* and diminished pharmacological actions of these drugs *in vivo* [1]. These changes qualitatively resembled those seen after administration of the group of inducers typified by phenobarbitone. In trying to characterize further the biochemical events which precede the onset of hepatic porphyria during HCB feeding [2], spectral studies and haem turnover studies associated with cytochrome P-450 have been made. That more than one form of cytochrome P-450 haemoprotein may exist has been suggested by the following studies: (a) a biphasic loss of radioactivity from the microsomal CO-binding particles [3, 4] (b) microsomal drug and ligand binding spectral studies [5–9] (c) electrophoretic studies of cytochrome P-450 [10, 11].

On the basis of spectral interactions of various ligands with cytochrome P-450, microsomal haem turnover studies and microsomal enzyme activities, it is evident that hexachlorobenzene causes a mixed type induction encompassing microsomal changes induced by both phenobarbitone and 3-methylcholanthrene.

MATERIALS AND METHODS

Animals

Female rats (80–140 g body wt) of the Agus strain were used throughout this study and were allowed free access to food and water.

Animal treatment

Hexachlorobenzene (0·2% w/w) was fed in the diet for 7–10 days prior to killing. Phenobarbitone sodium (50 mg/kg b.w.) dissolved in normal saline and 3-methylcholanthrene (20 mg/kg b.w.) dissolved in arachis oil were administered i.p. 16 and 40 hr prior to killing.

5-amino[4-^{14}C]laevulinate (sp. act. 53 mCi/m-mole, 0·75 or 3·0 μCi) was injected intraperitoneally and the animals killed at various intervals after the injection.

Isolation of microsomes and CO-binding particles

A 10% liver homogenate in 0·25 M sucrose was prepared and a post mitochondrial supernatant isolated after centrifugation of the homogenate at 9000 *g* for 20 min. Liver microsomes were isolated from the post mitochondrial supernatant by the calcium chloride–sucrose method [12] and were suspended in 0·1 M phosphate buffer pH 7·4 containing 1 mM EDTA so that 1 ml microsomes was equivalent to 200 mg original liver. Microsomal suspension (5 ml) was incubated with 0·2% lipase for 60 min at 37° in evacuated Thunberg tubes on a metabolic shaker. The mixture was centrifuged at 100,000 *g* for 60 min, the supernatant decanted and the pellet (CO-binding particles) resuspended in 3–4 ml 0·1 M phosphate buffer pH 7·4 containing 1 mM EDTA.

Haem crystallization and colour determination

One ml horse blood haemolysate (containing 6·3 mg haemin) was added as carrier to 2 ml CO-binding particles and haem crystallized by the strontium chloride/acetone method [13]. Haem was estimated as the pyridine haemochromogen [14] using an extinction coefficient of 34·4 $mM^{-1}\,cm^{-1}$ at 557 nm [15].

Radioactivity measurement

Radioactivity in CO-binding particles was measured in a Phillips liquid scintillation analyser PW 4510 with an efficiency of 72–78% utilizing INSTA-GEL as scintillant.

Radioactivity in haem (60–120 μg, dissolved in pyridine) was either measured in INSTAGEL with an efficiency of 30–46% determined by the addition of internal standard, or haem (in pyridine) was spotted on to a disc of tissue paper and pyridine allowed to evaporate for 3 hr. The [^{14}C]haem residue was then combusted to $^{14}CO_2$ in an Intertechnique Oxymat IN 4101, collected in a scintillation mixture containing 1% (w/v) 2,5 diphenyloxazole in 2-phenylethylamine–methanol–toluene–deionized water (33:22:40:5 by vol) and radioactivity counted with an efficiency of 70%.

Cytochrome estimations

Microsomal cytochromes P-450 and P-420 were routinely determined in 0·1 M phosphate buffer pH 7·4 as described previously [16] using a Unicam SP 1800. Both reference and test cuvettes were kept stoppered during the measurement of the cytochromes. In the assay of cytochrome P-420, carbon monoxide was bubbled into the test cuvette prior to the addition of sodium dithionite to both cuvettes. For accurate measurement of the peak wavelength of cytochrome P-450, a Cary recording spectrophotometer was used. An holmium filter was used to calibrate the latter recording spectrophotometer.

Microsomal ligand binding

(a) *Reduced pyridine.* Each cuvette contained 0·9 ml microsomal suspension (equivalent to 200 mg wet wt liver), 1·8 ml 0·1 M phosphate buffer pH 7·7 and a few milligrams of sodium dithionite. 0·3 ml water was added to the reference cuvette and 0·3 ml 0·5 M aqueous pyridine to the test cuvette. The difference in extinction, 424–500 nm and 446–500 nm were used as estimates of the 424 and 446 peaks respectively. (b) *Hexobarbitone and aniline.* Each cuvette contained (in 2·9 ml) microsomal suspension (equivalent to 130 mg original liver/ml) and 0·1 M phosphate buffer pH 7·4. Hexobarbitone and aniline in 0·1 M phosphate buffer pH 7·4 were added to the test cuvette and an equivalent vol of solvent to the reference cuvette. The concentrations of hexobarbitone and aniline in the cuvette were 1·9 mM and 10·1 mM final. The difference spectra induced by these drugs were recorded between 370–500 nm.

Protein estimation

Protein was determined by the method of Lowry *et al.* [17].

Special reagents

Hexachlorobenzene (organic analytical reagent grade) and phenobarbitone sodium were purchased from British Drug Houses Ltd., Poole, Dorset. 5-amino[4-^{14}C]laevulinate was bought from the Radiochemical Centre, Amersham, Bucks. Defibrinated horse blood was obtained from Burroughs Welcome, Beckenham, Kent. Lipase and 3-methylcholanthrene were purchased from Sigma Chemical Co., Kingston, Surrey.

RESULTS

Effect of hexachlorobenzene on the spectral characteristics of cytochrome P-450 in hepatic microsomes

Liver microsomes isolated from control rats (untreated or given arachis oil intraperitoneally) have a CO-difference spectrum with a peak at 450·5 nm. However, when rats are treated with HCB for 7–10 days (corresponding to maximal increase in cytochrome P-450) or 3-methylcholanthrene for 2 days, a wavelength shift occurs and the CO-difference spectrum reveals peaks at 448·7 and 448·4 nm respectively; in contrast, no shift in the wavelength peak is observed in microsomes isolated from phenobarbitone treated rats (Table 1).

Although CO combines with reduced microsomes to give only one spectral peak, a ligand such as pyridine gives two spectral peaks [18]; furthermore the two peaks appear to be in a pH-dependent equilibrium. The effects of inducer treatment upon the ratio of the two peaks at pH 7·7 are shown in Table 1. If HCB is fed for 7 days then the ratio is similar to that after phenobarbitone treatment. However, if feeding of HCB is maintained beyond 7 days, then the ratio is found to be greater than that seen with phenobarbitone.

Difference spectra can also be obtained after the addition of certain drugs to oxidized hepatic microsomes. Two main types of difference spectrum can be elicited and are known as Type I (hexobarbitone) and Type II (aniline) respectively. Addition of hexobarbitone to liver microsomes from HCB-treated rats produces a small increase in the Type I spectrum and addition of aniline to liver microsomes from HCB-treated rats increases markedly the magnitude of the Type II spectrum.

Disappearance of radioactivity from CO-binding particles in rats pretreated with hexachlorobenzene

The loss of radioactivity from the plasma and its appearance in the liver after 5-amino[4-^{14}C]laevu-

Table 1. Effect of hexachlorobenzene on the spectral characteristics of cytochrome P-450 in hepatic microsomes

	Peak wavelength (nm)	Pyridine spectrum (ratio of 446–424 nm peaks)	Hexobarbitone Type I spectrum	Aniline Type II spectrum
Control	450·5 ± 0·06 (7)	1·13 ± 0·04 (3)		
Hexachlorobenzene	448·7 ± 0·15* (5)	†7·32 ± 0·68 (3)	↑	↑
Phenobarbitone	450·3 ± 0·25 (4)	5·02 ± 1·07 (4)	↑	↑
3-Methylcholanthrene	448·4 ± 0·06* (4)	12·5	spectrum abolished	↑

Female rats of the Agus strain were fed normal powder diet or diet containing 0·2% (w/w) hexachlorobenzene for 10 days or given phenobarbitone sodium (50 mg/kg) or given 3-methylcholanthrene (20 mg/kg) i.p. 16 and 40 hr prior to killing. Difference spectra were recorded as described in the methods section. Results are expressed as Mean ± S.E.M. with number of observations in parentheses.

* $P < 0·01$ with respect to control.

† Ratio after 7 days feeding of hexachlorobenzene = 4·7.

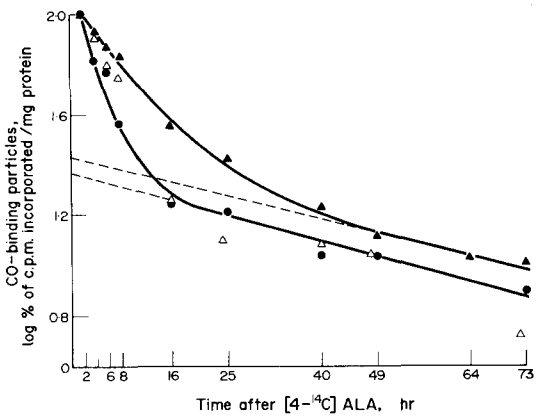

Fig. 1. Loss of radioactivity from the haemoprotein(s) of the CO-binding particles isolated from the livers of untreated and hexachlorobenzene-treated rats. Rats were fed normal powder diet or diet containing 0·2% (w/w) hexachlorobenzene for seven days and then 3 μCi 5-amino[4-^{14}C]laevulinate (57 nmoles) or 0·75 μCi (equivalent to 14 nmoles) was administered intraperitoneally and feeding of HCB continued throughout the duration of the experiment. Untreated and HCB-treated rats (1–3 rats per group) were killed at various intervals after isotope administration and CO-binding particles isolated from the liver microsomes. ●——●, Control; ▲——▲, HCB; △——△, Control (low 5-ALA dose).

linate administration is similar in untreated and HCB-treated rats. Radioactivity in liver microsomes reaches a maximum 30–60 min after isotope administration when approx 25 per cent of the total radioactivity in the liver is present in this fraction in both untreated and HCB-treated rats. After solubilization of the microsomes with lipase, only a small percentage (approx 10–15%) of total microsomal radioactivity is recovered in the soluble fraction. Almost all the radioactivity in the insoluble residue ('CO-particles') isolated from the livers of untreated and HCB-treated rats can be accounted for as haem. The loss of radioactivity with time is biphasic in both untreated and HCB-treated rats suggesting that the CO-particles contain at least two haemoprotein components (Fig. 1). The half-lives corresponding to the fast and slow phases have been determined by extrapolating the slow phase line to zero time and subtracting the slow phase values from the respective fast phase values. These calculations reveal half-lives of 3–4 and 45 hr for the corrected fast and slow components in untreated rats and half-lives of 7–8 and 49 hr in HCB-treated rats (Table 2).

Although several different groups have shown a biphasic loss of radioactivity from the CO-particles or microsomes of the livers of untreated rats, there is considerable variation in the half-lives of the two components especially the fast phase [3, 4, 19–22]. These variations have been ascribed to strain and age differences [20]. However, the variations may be related to the amount of isotopic 5-aminolaevulinate administered since if the conversion of 5-aminolaevulinate to haem is surplus to requirements, the excess haem may accumulate or be rapidly degraded to bilirubin. The loss of radioactivity from CO-particles isolated from untreated rats given 25 per cent (14 nmoles) of the original dose of 5-amino[4-^{14}C]laevulinate is shown in Fig. 1. There is still a biphasic loss of radioactivity from the CO-particles. This lower dose appears to give a slightly slower fast phase half-life ($t_{\frac{1}{2}}$ = 4–5 hr), but does not alter the half-life of the slow phase component ($t_{\frac{1}{2}}$ = 48 hr).

DISCUSSION

The number of foreign chemicals and drugs which possess the ability to cause hepatic microsomal enzyme induction is vast [23] and only two different patterns of enzyme induction have been described. The evidence on which compounds have been assigned to one of the two classes has been derived from:

(a) spectral interactions of ligands with microsomes and spectral characteristics of cytochrome P-450;

(b) changes in microsomal drug-metabolizing enzyme activities;

(c) microsomal haem turnover studies.

On the one hand, a group of chemicals typified by 3-methylcholanthrene and 3,4 benzpyrene increase in the liver:

(i) the amount of a microsomal haemoprotein cytochrome P-448 [8, 24]; and

(ii) the activities of a limited group of microsomal drug-metabolizing enzymes.

On the other hand, a larger group typified by phenobarbitone and chlordane increase in the liver:

(i) the amount of microsomal haemoprotein cytochrome P-450; and

(ii) the activities of a wide range of microsomal drug-metabolizing enzymes [25].

Table 2. Effect of hexachlorobenzene upon the amount of cytochrome(s) and upon the half lives of the haemoprotein components of the CO-binding particles

Treatment	Cytochrome P-420 nmoles/mg protein Mean ± S.E. (No of observations)	Half-lives ($t_{\frac{1}{2}}$) (hr)		Ratio of fast to slow fraction	Relative amounts	
		Fast corrected	Slow		Fast	Slow
Control	1·36 ± 0·07 (11)	3–4	45	4·9	1·13	0·23
HCB	1·77 ± 0·08 (12)	7–8	49	2·8	1·30	0·47

The details of this experiment are given in the legend to Fig. 1. Cytochrome P-420 was measured in the CO-binding particles isolated from the liver microsomes of these rats and the observations in each group pooled. The corrected half-life for the fast phase component was calculated as described in the text. The ratio of the fast phase haemoprotein to the slow phase haemoprotein was obtained by extrapolating the fast and slow phase lines to zero time, subtracting the slow phase zero intercept from the fast phase zero intercept and dividing the result by the slow phase zero intercept.

However, studies with HCB suggest that it shares some of the properties of both groups of inducers.

HCB pretreatment causes a change in the spectral characteristics of cytochrome P-450 by causing a peak shift to a lower wavelength similar to that seen after 3-methylcholanthrene, but not after phenobarbitone pretreatment [8, 24]. In contrast, changes in the duration of pharmacological action of hexobarbitone and zoxazolamine and increases in their metabolism *in vitro* by liver microsomes from HCB-treated rats [1] resemble those seen after phenobarbitone rather than 3-methylcholanthrene pretreatment [23].

Further spectral evidence involving the binding of ligands to microsomes shows that a typical Type I spectrum induced by hexobarbitone is elicited after HCB treatment which is quantitatively less than that seen after phenobarbitone and quite different from that after 3-methylcholanthrene (or 3,4 benzpyrene treatment) in which the Type I spectrum is lost [5, 26]. The magnitude of the spectral change induced after addition of aniline to liver microsomes is increased not only after HCB pretreatment but also after 3-methylcholanthrene and phenobarbitone pretreatment [5, 26].

In addition, the binding of pyridine to microsomes reduced with dithionite is altered after inducer treatment. When the ratio of the two spectral peaks at pH 7·7 is compared for each inducer it is found that the ratio after HCB treatment for 7 days corresponds closely to the ratio after phenobarbitone treatment. However, if the feeding of HCB is continued for 10 days the ratio is higher than after phenobarbitone treatment and may reflect a slow changeover from the phenobarbitone to the 3-methylcholanthrene pattern of induction. The appearance of two spectral peaks during binding of pyridine to reduced microsomes is analogous to that seen with ethylisocyanide [18] and appears to represent two haemoprotein components in a pH-dependent equilibrium [27].

Previous studies have shown that 5-aminolaevulinate is rapidly converted to haem and this haem is converted almost quantitatively to bile pigment without being reutilized [28, 29]. The administration of 5-amino[4-^{14}C]laevulinate to rats leads to the incorporation of radioactivity into hepatic microsomal haemoproteins, cytochromes P-450 and b_5. Cytochrome b_5 can be solubilized with lipase together with approx 50 per cent of microsomal protein leaving the labelled CO-binding haemoprotein (cytochrome P-420) in the insoluble residue [30]. A biphasic loss of radioactivity from CO-particles was observed, in agreement with previous studies [3, 4]. Since almost all the radioactivity could be accounted for as haem the presence of at least two haem containing components was indicated. There have been many measurements of the half-lives of the two components and considerable variability in the values reported [3, 4, 19, 21, 22]. Although these variations may be related to strain, sex or age differences, the amount of isotopic 5-aminolaevulinate administered varies considerably. A 4-fold difference in the amount of 5-amino[4-^{14}C]laevulinate administered intraperitoneally alters minimally the shape of the biphasic curve (Fig. 1) and tends to confirm that factors other than the dose of 5-aminolaevulinate administered determine the variability of the data reported previously.

During solubilization of microsomes with lipase, the typical cytochrome P-450 spectrum is gradually lost with a reciprocal increase in a peak at 420 nm (cytochrome P-420) [16, 30]. The nature and existence of cytochrome P-420 has recently been questioned since haem added to microsomers will give a P-420 spectrum [31]. Furthermore, *in vitro* studies in which [^{14}C]haem is added to microsomes and the mixture solubilized with lipase shows that despite the solubilization of 50 per cent of microsomal protein, almost all the radioactivity is associated with the CO-binding particles and this bound haem can give rise to a P-420 spectrum (Stonard, unpublished observations). It is impossible therefore to distinguish between haem bound to microsomes and degraded forms of cytochrome P-450 and P-448. However, radioactivity incorporated into CO-particles after isotopic 5-aminolaevulinate administration is associated almost exclusively with cytochrome and very little non-specific binding of haem to microsomes occurs [20, 32].

Although almost all microsomal haem can be accounted for by cytochromes P-450 plus b_5 [33, 34], there may exist a small pool of microsomal haem bound to microsomes which may quantitatively alter haemoprotein turnover. Furthermore, incorporation of radioactivity into the haem of microsomal cytochrome b_5 is slow and haem exchange between cytochrome b_5 and the other haem pool(s) may occur [35]. From the known time course of induction of cytochrome P-450 it is evident that the half-life of the fast phase component is too short to account for *de novo* synthesis of cytochrome P-450. Studies involving a comparison of the labelling of microsomal protein and haem suggest that apocytochrome P-450 [32] may exist in microsomes and is available to bind haem. Thus, in this study the biphasic loss of radioactivity from CO-particles is taken to represent turnover of two haemoprotein components derived from cytochrome P-450 species, but other interpretations can be made [19, 31].

Studies of the influence of inducer treatment upon liver microsomal haem turnover have been made by Levin and Kuntzman [3, 4]; these workers showed that 3-methylcholanthrene pretreatment did not alter the half-lives of either the fast or slow phase haemoprotein, but increased the amount of the slow phase haemoprotein [3]. In contrast, phenobarbitone or chlordane pretreatment increased the half-life of the fast phase haemoprotein and increased to an equal extent the amounts of both components [4]. HCB, like phenobarbitone and chlordane increases the half-life of the fast phase haemoprotein.

It seems probable that HCB and polychlorinated biphenyls (PCB's) which share certain structural characteristics, belong to the same class of microsomal enzyme inducers. Pretreatment of rats with Aroclor 1254* (a heterogeneous group of chlorinated biphenyls containing an av 54% chlorine) not only increases the amount of hepatic microsomal cytochrome P-450, but alters the spectral characteristics by shifting its peak to 448 nm [36]. Aroclor 1254 also increases the activities of a wide range of microsomal

* Monsanto Chemical Company.

drug metabolising enzymes [37–39]. Furthermore, HCB and the more chlorinated biphenyls produce a chronic hepatic porphyria in which the same pattern of porphyrins accumulate in the liver [2, 40].

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