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Mechanism of hexachlorobenzene-induced porphyria in rats

Effect of phenobarbitone pretreatment

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1. The effect of a pretreatment with phenobarbitone (PB) on the porphyrinogenic action exerted by hexachlorobenzene (HCB) was examined in female rats. 2. Kinetic studies of enzyme function after HCB poisoning showed that porphyrinogen carboxy-lyase was the only enzyme of haem biosynthesis that markedly lowered its activity. Both stages of uroporphyrinogen (UPG) III decarboxylation were decreased. This enzyme, together with UPG I synthase (increased levels) were the first enzymes altered. Subsequently, an increase in δ-aminolaevulinate (AmLev) synthase and ferrochelatase was detected; AmLev dehydratase was the last to increase. 3. On long-term exposure, PB alone did not modify the basal values of haem intermediates; only the content of cytochrome *P*-450 increased. All the enzyme activities studied showed no significant changes, except ferrochelatase, which increased. 4. With both drugs the metabolic impairment promoted by HCB was accelerated and enhanced by prior PB treatment, leading to the onset of an earlier and stronger porphyria. A more noticeable accumulation and excretion of higher carboxylated porphyrins and precursors was more promptly observed as a consequence of the early porphyrinogen carboxy-lyase blockade and the concomitant induction of AmLev synthase. Although the enzymic activities of both AmLev dehydratase and ferrochelatase were enhanced, this response differed in time. For UPG I synthase this pretreatment elicited lower values than those found in the HCB group. 5. Cytochrome *P*-450 contents were immediately and slightly enhanced by all the drugs, but the values for the combined treatment were the lowest. 6. Of the several hypotheses that could explain the action of HCB on the haem pathway, our results would suggest that the porphyrinogenic action of HCB is mediated by some of its metabolic products.

HCB, a polychlorinated hydrocarbon, causes marked disturbances in haem metabolism leading to the development of an hepatic porphyria of cutaneous type both in humans and in animals (De Matteis *et al.*, 1961; Cam & Nigogosyan, 1963; San Martín de Viale *et al.*, 1970). HCB in rats produces noticeable accumulation and excretion of uroporphyrin (eight carboxy groups) and heptacarboxyporphyrin (San Martín de Viale *et al.*, 1970, 1977; Taljaard *et al.*, 1972). These effects are a consequence of the marked decrease in the hepatic porphyrinogen carboxy-lyase produced by the drug (Elder *et al.*, 1976; San Martín de Viale *et al.*, 1976*a*) and they are accompanied by increased activities of AmLev synthase, AmLev dehydratase, porphobilinogenase and ferrochelatase (Rajamanickam *et al.*, 1972; Taljaard *et al.*, 1972; San Martín de Viale *et al.*, 1976*b*; Ríos de Molina *et al.*, 1977). Coproporphyrinogen oxidase was unaltered (Elder *et al.*, 1976).

The stimulation of drug-metabolizing systems by PB is well known (Conney, 1967). The barbiturate elicits many effects on the microsomes; it increases cytochromes *P*-450 and b_5, lipid, protein and RNA contents and induces different drug-metabolizing

Abbreviations used: AmLev, δ-aminolaevulinate; PBG, porphobilinogen; HCB, hexachlorobenzene; PB, phenobarbitone; UPG, uroporphyrinogen.

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enzymes (Wada *et al.*, 1968; Manchanahalli *et al.*, 1973). Its action on AmLev-synthase and ferrochelatase has been reported (Wada *et al.*, 1968; Kaufman *et al.*, 1970; Hasegawa *et al.*, 1970).

HCB, like other drugs, also increases the mixed-function-oxidase system (Wada *et al.*, 1968; Turner & Green, 1974; Mehendale *et al.*, 1975). Enzymic and electrophoretic studies had proved that HCB is an inducer of a cytochrome of the so-called 'mixed' type (Stonard, 1975; Vizethum *et al.*, 1980). In recent years numerous compounds were identified as metabolic products of HCB; pentachlorophenol, pentachlorothiophenol, tetrachlorohydroquinone, tetrachlorocatechol, tetrachlorothiophenol being the main ones (Mehendale *et al.*, 1975; Lui *et al.*, 1976; Koss *et al.*, 1976).

Taking into account all these findings, it would be interesting to ascertain the mechanism by which HCB produces its toxic response. The question arises as to whether its porphyria-inducing activity in mammals is due to the direct action of HCB or some active metabolite or reactive intermediate formed by the cell. With this goal, the combined effect of pretreatment with PB and HCB administration in haem metabolism was investigated.

The following parameters were measured: liver/body-weight ratio; levels and nature of porphyrins and precursors accumulated in livers and excreted by urine; cytochrome *P*-450 content; hepatic AmLev synthase, AmLev dehydratase, UPG I synthase, porphyrinogen carboxy-lyase and ferrochelatase activities as a function of HCB intoxication time and/or PB pretreatment.

Materials and methods

Chemicals

HCB [commercial grade; composition: HCB, 95%; tetra- and penta-chlorobenzene, 5% (w/w)] was generously given by Compañía Química S.A., Buenos Aires, Argentina. PB was a gift from Bayer Argentina S.A., Buenos Aires, Argentina. AmLev and PBG were purchased from Sigma. Uroporphyrin III was isolated from turacin (San Martín de Viale *et al.*, 1970). Protoporphyrin IX was obtained as described by Grinstein (1947). Porphyrinogens were prepared with sodium amalgam as described by Mauzerall & Granick (1958). The ion-exchange resins Dowex 1 (X8) and Dowex 50W (X8) (both 200–400 mesh) were A.G. grade from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Sephadex G-25 was purchased from Pharmacia, Uppsala, Sweden.

Animals

Female Wistar rats weighing 160–180g at the start of the experiment were housed individually in metabolic cages for collection of 24h urine and fed Purina 3 diet (Cabeca S.C.A., Buenos Aires, Argentina) and water *ad libitum*.

Animals were divided into four groups: (1) normal, untreated control; (2) treated with PB daily, in drinking water (0.1%, w/v); (3) treated with HCB administered daily by stomach tube (1g/kg body wt.); the drug was suspended in water (40mg/ml) containing Tween 20 (0.5ml/100ml); (4) treated with PB + HCB as in (2) and (3). PB was given 1 week before starting the HCB treatment and was continued throughout the experiment.

Rats from the four groups were killed by decapitation after weeks 1, 3, 5, 8 and 10 of HCB treatment. Livers were weighed and divided in two portions. (1) Homogenates were made with 0.154M-KCl (1:5, w/v) for estimation of porphyrin and cytochrome *P*-450 content and all the enzyme activities except AmLev synthase. They were centrifuged at 900*g* for 10min, then at 11000*g* for 20min to obtain mitochondria and at 105000*g* for 60min to obtain microsomes (microsomal fraction). In order to remove the endogenous porphyrins, homogenate supernatants (11000*g* or 105000*g*) from porphyric livers were filtered through a column (2.4cm × 30cm) of Sephadex G-25 with 0.134M-potassium phosphate buffer, pH7.0. The eluates with no or trace fluorescence were pooled and used as enzyme preparation. (2) Livers were homogenized in 0.9% (w/v) NaCl/0.5mM-EDTA/10mM-Tris/HCl buffer, pH7.4 (1:3, w/v) for determination of AmLev synthase activity. All procedures were carried out at 4°C.

Assay of hepatic porphyrins and cytochrome *P*-450 as well as enzymic studies were performed simultaneously in each animal on the same day, in duplicate. The whole experiment was repeated twice. To study AmLev dehydratase and porphyrinogen carboxy-lyase in postmicrosomal supernatant, a third group of animals was used.

Hepatic and urinary content of porphyrins and precursors

Assays for AmLev, PBG and porphyrins were performed weekly in 24h urine specimens. They were determined in 0.3–1ml samples of urine essentially by the method of Piper *et al.* (1973). Two separate columns were used; PBG was eluted from a column of Dowex 1 with 1M-acetic acid and AmLev was eluted from a column of Dowex 50 with 1M-sodium acetate after removing urea. AmLev pyrrole and PBG were measured colorimetrically by the method of Mauzerall & Granick (1956). Porphyrins were eluted from the Dowex 1 column with 10% (w/v) HCl and determined spectrophotometrically by using the Rimington & Sveinsson (1950) correction formula. Absorption

coefficients for total free porphyrins, calculated as average values and taking into account the percentage of each component present in the mixture, were used (Tomio *et al.*, 1970). Recovery of AmLev, PBG and porphyrins by these procedures were 95, 98 and 90% respectively.

Porphyrin content in liver was determined in 0.1–3ml portions of whole homogenates as total free porphyrins in 5% (w/v) HCl as described by San Martín de Viale *et al.* (1977). Porphyrin methyl esters were separated by Falk & Benson's (1953) method and determined spectrophotometrically as described by Tomio *et al.* (1970).

Enzyme activities

The hepatic activity of AmLev synthase was assayed in whole homogenates by the method of Marver *et al.* (1966) and expressed as nmol of AmLev formed/h per g of liver by using an ε^{mM}_{555} of 62. The incubation mixtures, containing 0.1M-glycine, 0.01M-EDTA, 0.08M-Tris/HCl buffer, pH7.2, and 0.5ml of homogenate in a final volume of 2ml, were incubated at 37°C for 60min.

AmLev dehydratase was assayed by the method of Gibson *et al.* (1955). Assay mixtures contained (in 2ml) 3.75mM-AmLev, 12.5mM-cysteine, 67mM-potassium phosphate buffer, pH7.0, and 0.05ml of postmitochondrial or postmicrosomal supernatant. Preincubation for 60min at 37°C before substrate addition and an incubation period of 60min were used.

For the determination of UPG I synthase activity, incubation mixtures, containing (in 60μl) 0.1M-sodium phosphate buffer, pH7.5, 0.2mM-PBG and, as enzyme preparation, 10μl of either postmitochondrial supernatant for normal and PB-treated rat livers or 20μl of Sephadex G-25 eluates for porphyric livers, were used. Assays were incubated aerobically at 37°C for 60min in the dark. Fluorimetric determination of the porphyrins produced was as described previously (Kreimer-Birnbaum & Tomio, 1976).

Liver porphyrinogen carboxy-lyase activities in either postmitochondrial and postmicrosomal supernatants, from normal and PB-treated rat livers or in Sephadex G-25 eluates from porphyric livers, were measured as described by San Martín de Viale *et al.* (1977). The reaction mixtures contained (in 3ml) 67mM-potassium phosphate buffer, pH7.0, 1mM-reduced glutathione, 1mM-EDTA, 2μM-UPG III and, as enzyme preparation, 0.25ml of supernatant or 0.75ml of the eluates. The assays were incubated anaerobically in Thunberg tubes in the dark at 37°C for 30min. The first stage of porphyrinogen carboxy-lyase activity, i.e. UPG decarboxylation is expressed as hepta- + hexa- + penta- + tetra-carboxyporphyrins formed/30min per mg of protein. The second stage of porphyrinogen carboxy-lyase activity, i.e. coproporphyrinogen formation, is expressed as tetracarboxyporphyrin formed/30min per mg of protein (García *et al.*, 1973).

Ferrochelatase activity was measured in mitochondrial fractions. The 11000*g* pellets were frozen at −20°C for up to 24–48h, and, upon thawing, portions equivalent to 1g of liver were adjusted to 1.4ml with incubation buffer. Enzyme activity was measured as described by Porra & Jones (1963), including a 5min enzyme preincubation with protoporphyrin IX. The incubation mixture contained (in 3.2ml) 100nmol of protoporphyrin IX, 200nmol of ferrous sulphate, 5mM-succinate, 50mM-Tris/HCl buffer, pH8.2, and 0.8ml of enzyme preparation. Incubations were carried out at 37°C aerobically in the dark.

Liver cytochrome P-450

The concentration of cytochrome *P*-450 in microsomes was determined by the method of Omura & Sato (1964), from the CO difference spectrum of dithionite-reduced samples, using $\Delta\varepsilon^{mM}_{450-490} = 91$.

Protein determination

Proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Statistical treatment of results

Results are expressed as the arithmetic means ±S.E.M., and the means were compared by Student's *t* test. To obtain the value of *P* a degree of freedom $n_1 + n_2 - 2$ was used throughout.

Results

Urinary excretion of precursors and porphyrins

Urine was collected weekly from each animal in all the four groups (normal, PB, HCB and PB+HCB) and the contents of AmLev, PBG and porphyrins were determined to detect the onset of the porphyria. As shown in Fig. 1, PB treatment did not modify the normal levels of AmLev (23±1.7μg/24h), PBG (8.6±0.7μg/24h) and total porphyrins (2.2±0.2μg/24h) excretion. Animals receiving both treatments showed enhanced levels of these compounds from week 4, porphyrin excretion always preceding that of the precursors. HCB-treated rats also showed an increased excretion of both precursors and porphyrins, but this was achieved later. Urinary content rose to 600μg/24h for AmLev, 3500μg/24h for PBG and 145μg/24h for total porphyrins. The increase in porphyrins was observed from week 5 to week 6 and preceded by about 2weeks that of precursors.

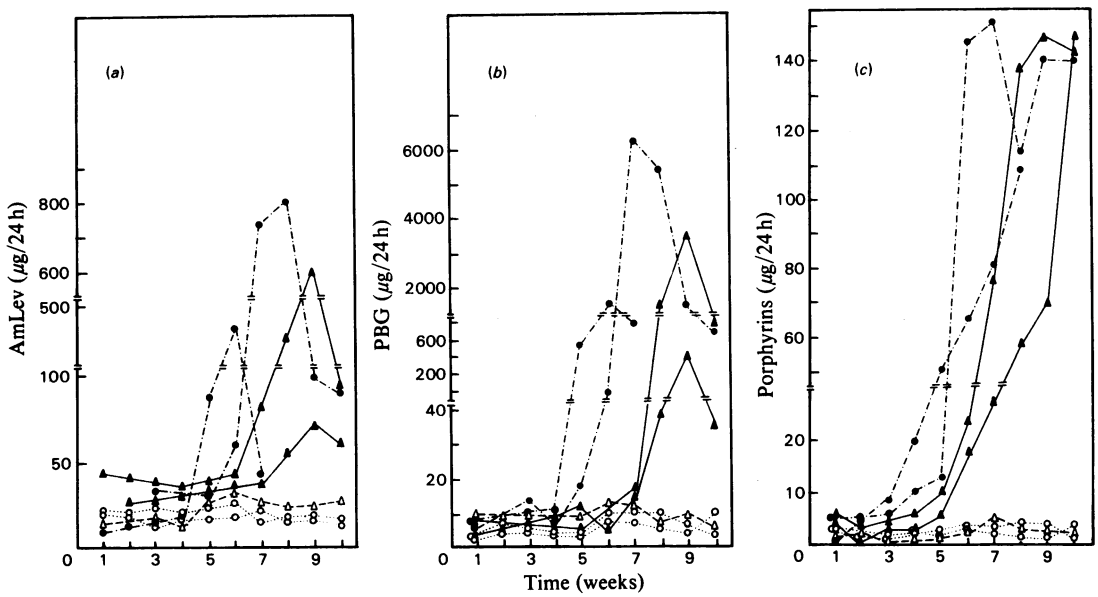

Fig. 1. *Urinary AmLev (a) PBG (b) and porphyrin (c) excretion by normal or treated rats*
Female rats were treated with PB (1 g/litre in drinking water) 1 week before the administration of HCB (1 g/kg daily) and throughout the experiment. HCB was given by gastric intubation from week 0. Porphyrins and precursors were estimated as described in the Materials and methods section. Each point represents urinary excretion of individual and characteristic rats of each group for animals treated with PB + HCB (●), HCB (▲), PB (○) and the mean value from five rats for controls (△).

Higher values for rats treated with both drugs were generally observed and preceded those attained in HCB-poisoned rats.

Normal and PB-treated rats excreted coproporphyrin (four carboxy groups) as the major urinary tetrapyrrole compound throughout. As shown in Fig. 2, PB + HCB and HCB groups also showed similar pattern of excretion until weeks 2 and 4 respectively. The subsequent coproporphyrin decrement was paralleled by a concomitant increment of highly carboxylated porphyrins, mainly uroporphyrin and heptacarboxyporphyrin (Figs. 2*a* and 2*b*). It is worth pointing out that the pattern of porphyrin excretion remains constant between weeks 7 and 10 of treatment for the animals receiving both drugs. The same behaviour was observed between week 8 and week 10 in rats treated only with the porphyrinogenic drug.

Liver weight and hepatic porphyrin accumulation

A significant increase in the ratio of liver to body weight, even from the first week and throughout the treatment period, was found in all treated groups.

The higher values were elicited by rats pretreated with PB (from 5.5 to 6.8 g liver wt. per 100 g body wt). The extent of increase was always PB + HCB > HCB > PB > normal. Whereas untreated rats exhibited a constant ratio (about 3.6) during the 10 weeks, treated rats only maintained a constant but higher ratio up to the week 5; after that a considerable increase was noted.

Rats pretreated with PB exhibited an hepatic accumulation of porphyrins as early as the week 3 of poisoning (8 μg/g liver), whereas the HCB-treated group showed an increased level (15 μg/g liver) only 2 weeks later (Fig. 3).

The PB-treated group showed a porphyrin concentration no different from that in untreated rats (1.94 ± 0.39 μg/g of liver). Starting from week 3 and until the end of the experiments, mean levels were always significantly higher ($P < 0.05$) in PB-pretreated rats (maximum 600 μg/g of liver) than in HCB-treated rats (maximum 300 μg/g of liver). Although increasing levels were observed in this last group throughout the present study, a plateau was reached after week 8 in the combined treatment.

Only traces of copro-and proto-porphyrins were detected in the livers of control and PB-treated groups. In both HCB-treated groups the same pattern of distribution was observed until week 1, after which a change to more-carboxylated porphyrins was noted, being achieved early in the pretreated rats. At week 3 the porphyrin accumulation in PB + HCB-treated rat livers showed uroporphyrin (50–60%), heptacarboxyporphyrin (40–30%) and only traces of hexa- and penta-

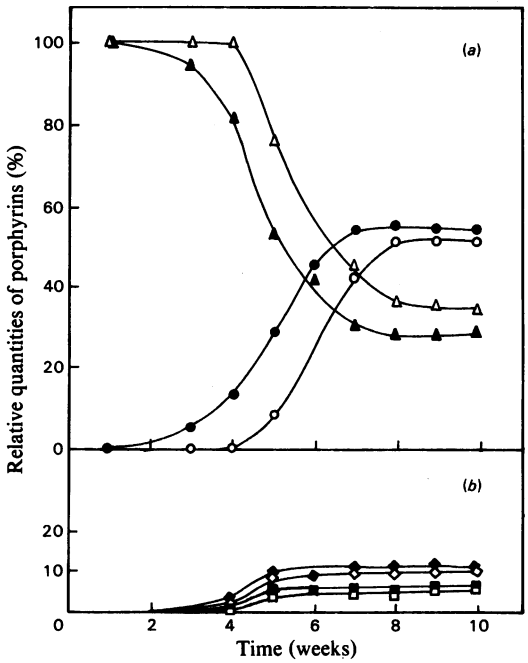

Fig. 2. *Nature of urinary porphyrins in HCB- and PB+HCB-treated rats*

Treatment conditions were as described in Fig. 1. Urinary porphyrins from characteristic rats were eluted, esterified and chromatographed by the methods described in the text. Relative quantities were spectrophotometrically determined and represented as coproporphyrin (△, ▲), uroporphyrin (○, ●), heptacarboxyporphyrin (◇, ♦), and penta- + hexa- carboxyporphyrins (□, ■). Open symbols correspond to the HCB-treated group and closed symbols to the PB+HCB-treated group.

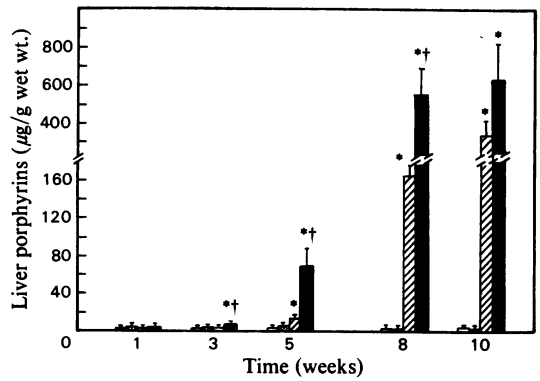

Fig. 3. *Effect of PB pretreatment on hepatic porphyrin accumulation by HCB*

Treatment conditions were as described in Fig. 1. Rats were killed at various times, livers excised and porphyrins isolated. Total free porphyrin contents were measured in 5% (w/v) HCl as indicated in the Materials and methods section. Data are expressed as means ± S.E.M. for five to six rats. ■, PB+HCB-treated; ▨, HCB-treated; ⊡, PB-treated; □, normal animals. $P<0.05$ when compared with normal(*) or with the HCB-treated group (†).

carboxyporphyrins. From then on the percentage of uroporphyrin increased to 70–75% at the expense of heptacarboxyporphyrin (30–25%). Hexa- and penta-carboxyporphyrins were not detected. These ratios were not modified with longer treatment. A similar distribution pattern was found for the HCB-treated group but 2 weeks later.

Enzyme activities

(*a*) *AmLev synthase.* In the two HCB-treated groups, hepatic AmLev synthase activity was significantly increased (Fig. 4), whereas in animals treated only with PB this activity did not differ significantly from that in control rats. For animals treated with PB+HCB a statistically significant increase ($P<0.05$) in this activity was already observed by week 3. Then there was a sharp rise, reaching a maximum at week 8, representing 5.6 times the normal mean value. A similar but 2-weeks-retarded time course was observed in the HCB group, which showed a slower rate of AmLev synthase and the maximum value attained represented an increase of only 3.6 times the normal mean value.

(*b*) *AmLev dehydratase.* Liver AmLev dehydratase activity showed a biphasic response with the treatment time in HCB- and in PB+HCB-treated groups (Fig. 5). Up to week 3 both groups exhibited activities the same as, or slightly below, those in untreated controls, followed by an increase, to a different extent for each group, until the same level was reached at the end of the experiment. This value is about twice that in control animals. The greatest responses in both phases were always found in rats pretreated with PB.

The group treated with PB alone showed lower-than-normal AmLev dehydratase activity throughout the experiment. We therefore considered it worthwhile to investigate whether this decrease was due either to a direct action of the drug on this enzyme or to increased microsomal protein content. To check these possibilities, 105000*g* and 11000*g* supernatants were compared. With post-microsomal supernatants of PB-treated rats, no significant difference from untreated controls was observed. Moreover, the microsomal supernatants showed the highest responses for each treated group.

(*c*) *UPG I synthase.* Hepatic UPG I synthase activities in the group receiving only PB remained

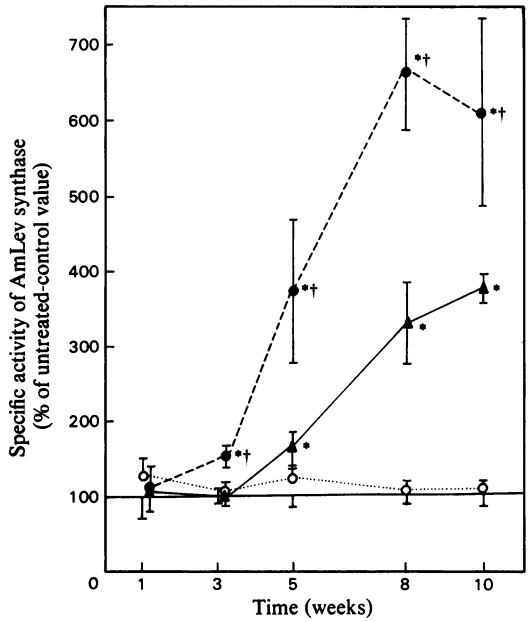

Fig. 4. *Effect of drug administration on AmLev synthase*
Treatment conditions were as described in Fig. 1. Rats were killed at different times and livers immediately removed, homogenized and AmLev synthase activity measured as described in the Materials and methods section. The basal activity of AmLev synthase in control animals was estimated as 14.7 ± 4.2 nmol of AmLev/h per g of liver and was taken as 100% for the determination of relative specific activities of the enzyme in PB + HCB (●)-, HCB (▲)- and PB (○)-treated groups. Each point represents the mean for six animals. $P < 0.05$ when compared with normal (*) or with the HCB-treated group (†).

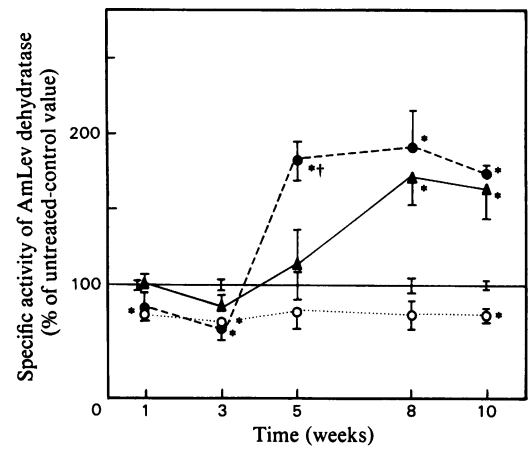

Fig. 5. *Effect of drug administration on AmLev dehydratase*
Treatment conditions were as described in Fig. 1. At various times, rats were killed, livers removed and homogenized as described in the text. Protein (1–1.4 mg) from the 11000*g* supernatant were used to measure AmLev dehydratase activity. Relative specific activities are expressed as percentages of the control value (14.7 ± 1.2 nmol of PBG/h per mg of protein) for PB + HCB (●), HCB (▲) and PB (○). Each point represents the mean ± S.E.M. for six animals. $P < 0.05$ when compared with normal (*) or with the HCB-treated group (†).

essentially unchanged during the course of experiments (Fig. 6). In contrast, the two groups of rats exposed to the porphyrinogenic drug showed slight, but significant, increased activities even from the first week. The levels attained were no more than twice the normal values. No important differences between the two groups were found. In spite of this, barbiturate-pretreated rats elicited lower values than those treated with HCB alone.

(*d*) *Porphyrinogen carboxy-lase.* HCB and PB + HCB treatment promoted decreases in the first stage of porphyrinogen carboxy-lyase activity, even from week 3 (Fig. 7), values being 17% and 40% respectively when compared with untreated controls. At longer times the hepatic activities were markedly reduced, reaching a constant value after week 8 (decrease of 58 and 75%). Again, the activities of rats receiving both drugs were the lowest, and the same diminished levels as obtained

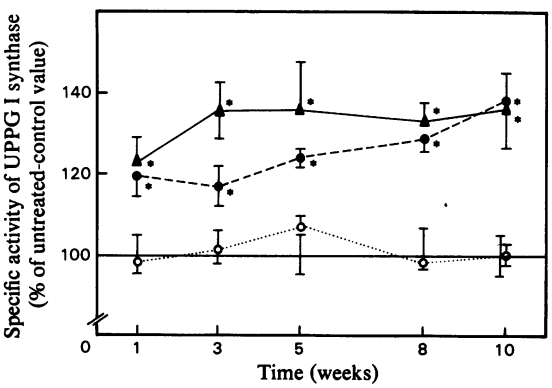

Fig. 6. *Effect of drug administration on UPG I synthase*
Animals were treated as in Fig. 1. Rats were killed at the time indicated and the liver homogenized. UPG I synthase activity was measured with PBG as substrate in the 11000*g* supernatant [for control and PB (○) groups] or in the Sephadex G-25 eluate [for PB + HCB (●) and HCB (▲) groups] as described in the Materials and methods section. Protein incubated from supernatant and eluate ranged from 0.25 to 0.32 mg and from 0.11 to 0.20 mg respectively. Relative specific activities are expressed as a percentage of that of control animals (43.7 ± 0.98 pmol of porphyrins/h per mg of protein). Each point represents the mean ± S.E.M. for four animals. $P < 0.05$ when compared with controls (*).

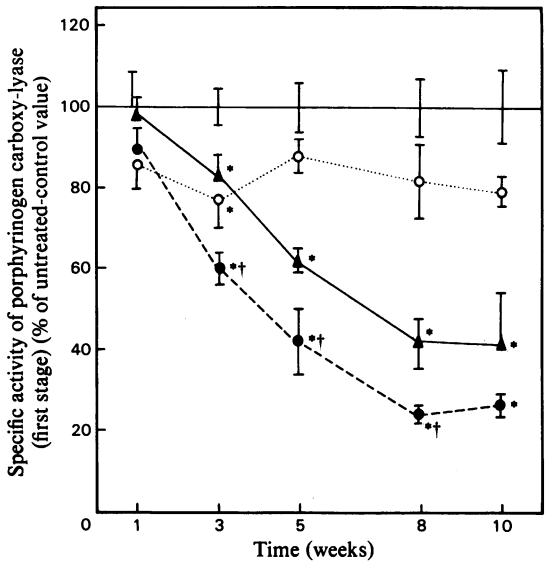

Fig. 7. *Effect of drug administration on porphyrinogen carboxy-lyase (first stage)*

Treatment conditions were as described in Fig. 1. At various times rats were killed, livers homogenized and the 11000*g* supernatant of control and PB (○)-treated rats or Sephadex G-25 eluates of PB+HCB (●)- and HCB (▲)-treated rats were used as enzyme source. The first stage of porphyrinogen carboxy-lyase, i.e. UPG decarboxylation, was measured as the formation of hepta-+hexa-+penta-+tetra-carboxyporphyrinogens, with UPG III as substrate. The amount of protein incubated ranged from 7 to 10mg always. Relative specific activities are expressed as percentages of the control value (0.62 ± 0.01 nmol of porphyrins/30min per mg of protein). Each point represents the mean ± S.E.M. for five animals. $P < 0.05$ when compared with normal (*) or with the HCB-treated group (†).

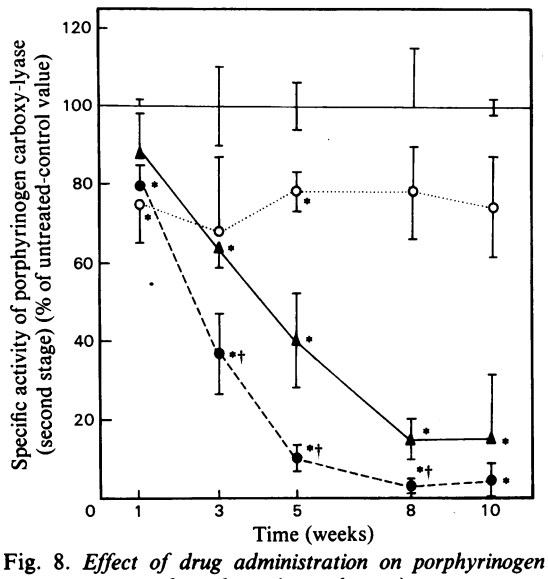

Fig. 8. *Effect of drug administration on porphyrinogen carboxy-lyase (second stage)*

Treatment and experimental conditions were as in Fig. 7, except that the second stage of porphyrinogen carboxy-lyase activities, i.e. coproporphyrinogen formation, was measured. The postmitochondrial supernatant of normal and PB (○)-treated rats or Sephadex G-25 eluates of PB+HCB (●)- and HCB (▲)-treated groups were used. Relative specific activities are expressed as percentages of control values (0.22 ± 0.01 nmol of tetracarboxyporphyrin/30min per mg of protein). Each point is the mean ± S.E.M. for five animals. $P < 0.05$ when compared with the normal (*) or with the HCB-treated group (†).

in HCB-treated groups were obtained 2–3 weeks beforehand. For the second stage of decarboxylation (coproporphyrinogen formation) (Fig. 8) similar kinetics were found in both HCB-treated groups. The response was again time- and pretreatment-dependent. Thus the decline was always fastest and highest in the PB-pretreated rats. Values in enzyme activities lower than 50% of control were noted at weeks 5 and 3 for the HCB and PB+HCB groups respectively. In fully developed HCB poisoning minimum values were reached (85% or 97% for HCB- or PB+HCB-treated groups compared with normal values) and remained constant.

In the PB-treated group, first and second stages of decarboxylation were diminished by nearly 20–30% during the course of the experiments. As in the study carried out for AmLev dehydratase, activities of porphyrinogen carboxy-lyase in 105000*g* supernatants were compared. The significant decreases in both stages of decarboxylation, found in postmitochondrial enzyme of PB-treated rats, disappeared when postmicrosomal fractions were used. In addition, activities in the PB+HCB-treated group measured with postmicrosomal supernatant and eluted from Sephadex G-25 were always lower than those of the HCB-treated group.

The data obtained with the postmicrosomal supernatants for AmLev dehydratase and porphyrinogen carboxy-lyase show that an apparent enzyme inhibition produced by the barbiturate should be disregarded. The decreases observed are probably due to the increased microsomal protein present in the 11000*g* supernatants.

(*e*) *Ferrochelatase.* As shown in Fig. 9, all drugs assayed increased the activity of hepatic ferrochelatase, but their kinetics differed. Animals exposed to barbiturate showed essentially constant, but noteworthy, increments of enzyme activity (nearly twice the normal mean values). This effect was evident as early as the first week.

For both groups treated with HCB there was a

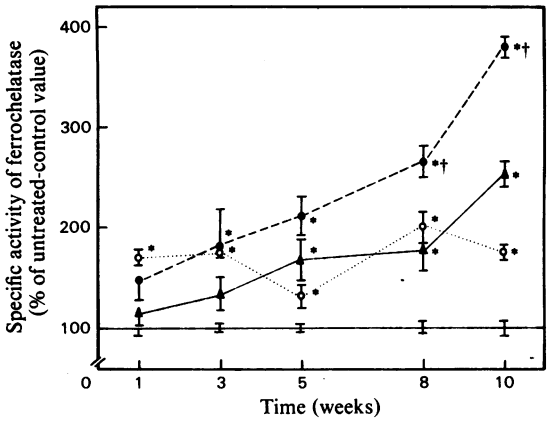

Fig. 9. *Effect of drug administration on ferrochelatase*
Treatment conditions were as described in Fig. 1. Ferrochelatase activity was measured with protoporphyrin IX as substrate in mitochondria from rat liver obtained as indicated in the Materials and methods section. The amount of protein incubated ranged from 11 to 19mg in the assays. Relative specific activities are expressed as percentages of control values (0.92±0.05nmol of haem/30min per mg of protein) for PB+HCB (●)- and HCB (▲)-treated groups and PB (○). Each point represent six rats. $P<0.05$ when compared with normal (*) or with HCB group (†).

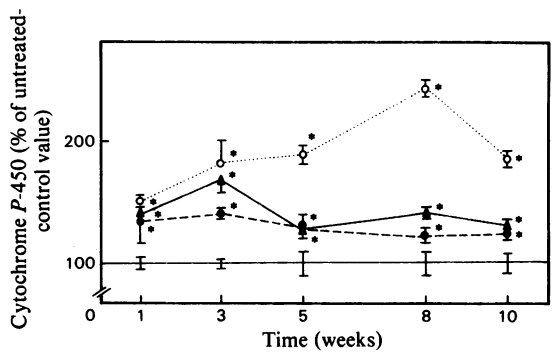

Fig. 10. *Effect of drug administration on cytochrome P-450 concentration*
Treatment conditions were as in Fig. 1. Rats were killed at different times, the liver removed and microsomes obtained as described in the Materials and methods section. Cytochrome *P*-450 contents were measured and results (mean±S.E.M.) are given as percentages of the control value (0.71±0.04nmol/mg of microsomal protein) for six animals. ●, PB+HCB-treated; ▲, HCB-treated; ○, PB-treated. $P<0.05$ when compared with normal animals (*).

progressive increase throughout in ferrochelatase activity, but at different times, being later (week 5) for rats exposed to HCB alone. Again, ferrochelatase activity in groups treated with both drugs was the highest and was noteworthy at the end of the treatment.

Cytochrome P-450

In this long-term experiment hepatic cytochrome *P*-450 showed an immediate and slight (about 2-fold) increase in its content as a consequence of the action of assayed drugs (Fig. 10). A later relative decrease was noted in all the groups, except the PB-treated group. The maximum values obtained for livers of rats treated with the barbiturate were 2.4 times normal mean values. There were no significant differences in cytochrome content between animals of the HCB- and the PB+HCB-treated groups, but the values obtained for the last group were always lower than for the first one.

Discussion

The results of the present investigation indicate that chronic HCB administration to female rats promotes increases, to different extents, in AmLev synthase, AmLev dehydratase, UPG I synthase and ferrochelatase activities. Porphyrinogen carboxy-lyase is the only enzyme of the haem pathway that progressively and markedly diminishes its activity in both stages of UPG III decarboxylation. Notwithstanding the early diminution of porphyrinogen carboxy-lyase activity, neither accumulation of liver porphyrins nor their urinary excretion are observed until 5 weeks after treatment. Decreases near to, or higher than, 50% in the UPG III decarboxylation process must be reached to impair the regulation of the haem pathway, allowing the biochemical manifestation of this enzyme failure. These decreases were closely correlated with the increase in AmLev synthase, which promoted a porphyrinogenic action still earlier in the PB-pretreated group. It is interesting to point out that decreases near to 50% in the activity of human porphyrinogen carboxy-lyase without clinical manifestation of porphyria were reported in latent cases of porphyria cutanea tarda (de Verneuil *et al.*, 1978).

Urinary porphyrin excretion always precedes that of its precursors, owing to the primary action of HCB on porphobilinogenase and porphyrinogen carboxy-lyase.

The biphasic curve of cytochrome *P*-450 content obtained in all treatments can be ascribed to two different effects. In the first phase, at short time exposure, all assayed drugs enhance the microsomal protein content, even before significant changes in the activities of most of the studied enzymes are observed. This elevation without a concomitant increase in AmLev synthase may be

due to a primary effect of the drug on the rate of apocytochrome *P*-450 synthesis (Rajamanickam *et al.*, 1972). In the second phase, the decrease of the cytochrome *P*-450 content is observed even in the presence of PB. The striking diminution of porphyrinogen carboxy-lyase activities would deplete the haem pool, which tightly controls haemoprotein synthesis, making this enzyme the rate-controlling factor. Moreover, it would seem that cytochrome *P*-450 levels reached with HCB cannot be attained in rats pretreated with PB, since a higher diminution in the decarboxylation process is detected beforehand. Puzynska *et al.* (1979), working with rather similar conditions of drug poisoning but over 17 days, detected levels of cytochrome *P*-450 in agreement with our studies. In animals treated with PB alone, the cytochrome *P*-450 content keeps up its enhanced level, since the barbiturate does not impair the decarboxylation process. Enhanced ferrochelatase activity can also play some role in this increase.

The results with both drugs show that the metabolic impairment promoted by HCB is accelerated and enhanced by prior PB treatment, leading to the onset of an earlier and stronger porphyria. Thus this pretreatment allows the pattern of accumulation and excretion of haem intermediates to be modified 2 weeks earlier and to increase both hepatic porphyrins and urinary PBG twofold. These effects reflect the more drastic alteration of the two key enzymes. The stronger porphyrinogen carboxy-lyase decrease does not allow an adequate supply of haem, leading to an early induction of AmLev synthase with the consequent pattern of accumulation and excretion of higher carboxylated porphyrins. Still, the higher imbalance found between the increased levels of AmLev synthase and UPG I synthase, making the last enzyme more limiting than in HCB poisoning, may explain the massive and 2-weeks-early excretion of PBG.

All these findings complement our previous reports (Graziano *et al.*, 1976; Ríos de Molina *et al.*, 1976) and are consistent with the observation of Kerklaan *et al.* (1979).

There are several hypotheses accounting for the porphyrinogenic action of HCB, but the true mechanism is unknown. Among them are: (i) a direct action of HCB or of some active metabolite(s) or reactive intermediate(s) formed by the cell; (ii) an indirect effect exerted by each one of these compounds; (iii) a synergistic effect of them. Our observation that the same levels of AmLev synthase, porphyrinogen carboxy-lyase, cytochrome *P*-450 and haem intermediates are reached early in animals pretreated with PB may suggest that the inducing action is mediated through the formation of an active metabolite. This early response can be ascribed to the prior treatment producing an induction of the drug-metabolizing system and accounts for a greater biotransformation of HCB, leading to a faster response. Then, the initial action of the active metabolite on porphyrinogen carboxy-lyase, namely inhibition or repression, may be to alter the controlling mechanism of the haem pathway.

From the observation that in the first week, where the impairment of porphyrinogen carboxy-lyase is not important, the levels of cytochrome *P*-450 in animals treated with PB + HCB were lower than those of PB and HCB alone, it is possible to infer that (i) an interaction of the halogenated drug with the haemoprotein or with some of its components or in the coupling process, take place (Unseld & De Matteis, 1978; Ortiz de Montellano & Mico, 1981) and (ii) such action could be mediated by a metabolite.

In support of our suggestion of an action mediated through a metabolite are the following observations: (1) Agus rats, more susceptible to the porphyrinogenic drug than the Wistar strain, do have a higher liver concentration of its metabolites (Debets *et al.*, 1981); (2) pentachlorothiophenol is found to be able to alter hepatic porphyrin metabolism in female Wistar rats (Koss *et al.*, 1977); (3) pentachlorophenol, one of the main HCB metabolites, is able to accelerate the onset of the porphyria by HCB (Debets *et al.*, 1980).

However, it is possible to suppose that the porphyrinogenic action of HCB is exerted *per se* and that the results obtained by administration of PB are the consequence of a synergistic action of both drugs on the metabolic pathway of hepatic haemoprotein. It was reported that metabolites such as pentachlorophenol (Goldstein *et al.*, 1977; Wainstok de Calmanovici & San Martín de Viale, 1980), pentachlorothiophenol, pentachlorothioanisol, its sulphoxide and sulphone, do not induce porphyria in female rats (Koss *et al.*, 1979). Nevertheless, it is likely that the metabolites can contribute indirectly to the toxic effects of HCB. Studies with HCB *in vitro* demonstrate that this drug does not exert a direct action on the key enzyme porphyrinogen carboxy-lyase (Ríos de Molina *et al.*, 1980).

According to the foregoing facts the porphyrinogenic action of HCB could still be carried out by some of its metabolites different from those already assayed, and among them epoxides, radicals or other short-half-life reactive metabolites.

The exacerbating effect on the HCB porphyria accomplished by diethyl maleate (Kerklaan *et al.*, 1979), the detoxificant role of glutathione that decreases the state of porphyria produced by lower chlorinated benzenes (Rimington & Ziegler, 1963), the label arising from HCB bound to macromole-

cules such as protein (Koss *et al.*, 1980), and our own results, would support the formation of such toxic metabolite in the biotransformation of HCB and its role in the onset of this porphyria.

However, a combined action of drug and metabolites, as was demonstrated for pentachlorophenol plus HCB (Debets *et al.*, 1980), and/or action of HCB *in vivo*, PB acting synergistically, cannot be discarded.

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