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HEPATIC DRUG METABOLISM AND HAEM BIOSYNTHESIS IN LEAD-POISONED RATS

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1 Pretreatment of rats with intraperitoneal injections of lead was shown to result in a depression of the microsomal mixed function oxidase system, as assessed by a decrease in hepatic microsomal P-450 and b_5 content and by a decrease in the activity of the enzymes aniline hydroxylase and aminopyrine demethylase. Lead had a more marked effect on cytochrome P-450 than b_5.

2 The activity of the rate-limiting enzyme of haem biosynthesis, δ-aminolaevulinic acid synthase, was inversely correlated with the microsomal cytochrome P-450 content.

3 The activity of the haem biosynthetic enzymes δ-aminolaevulinic acid dehydratase, coproporphyrinogen oxidase and ferrochelatase were decreased by increasing lead pretreatment.

4 The activity of the haem catabolic enzyme, haem oxygenase, was increased by lead pretreatment.

Introduction

It has long been recognized that haem synthesis in man is severely affected by increased body lead burden. This inhibition of haem production is well known to result in decreased levels of circulating haemoglobin in lead-exposed individuals. The possibility of altered drug metabolism due to inhibition of synthesis of the microsomal haemoprotein cytochrome P-450 has been the subject of a number of studies both in man and animals.

Recent reports (Alvares, Kapelner, Sassa & Kappas, 1975; Meredith, Campbell, Moore & Goldberg, 1977) of human studies have shown that in acute lead intoxication in man there is a depression in the elimination rates of phenazone (antipyrine), a drug metabolized by the liver cytochrome P-450-dependent microsomal mixed function oxidase system. This interpretation was supported by the finding of decreased half lives and increased clearance of the drug following chelation therapy. Chelation therapy was also shown (Meredith *et al.*, 1977) to result in a fall in blood lead levels and a rise in haemoglobin and erythrocyte delta-aminolaevulinic acid (ALA) dehydratase activity, an intermediate enzyme of the haem biosynthetic pathway.

Results of studies in animals have produced contradictory results. Preliminary studies by Ribeiro (1970) on the effects of arsenic, beryllium, lead and mercury on mouse liver drug metabolizing enzymes indicated that pretreatment of mice with lead nitrate to give liver levels of 10^{-4} and 10^{-6} M did not alter hexobarbitone sleeping times; nor were the *in vitro* microsomal enzyme activities, which were measured, affected. When lead was added to control microsomes at concentrations between 10^{-3} and 10^{-5} M, no inhibition of hexobarbitone oxidase was noted. In contrast the results of Alvares, Leigh, Cohn & Kappas (1972) and Scoppa, Roumengous & Penning (1973) suggest that lead has a significant effect on microsomal drug metabolism. Alvares *et al.* (1972) found a 40 to 50% decrease in metabolism of drugs by hydroxylation and demethylation and a significant decrease in cytochrome P-450 levels and an increase in hexobarbitone sleeping times, 24 h after the intravenous injections of lead chloride. From the results of their studies the authors considered that lead exerts its effect on drug metabolism via an inhibition of haem synthesis.

Scoppa *et al.* (1973) attempted to verify this hypothesis by correlating the inhibition of blood and liver ALA dehydratase by lead with drug metabolism. They were able to demonstrate lowered activity of this enzyme and a depression in cytochrome P-450 levels and an associated depression of *in vitro* activities of the microsomal mixed function oxidase system.

Despite these studies which give indirect evidence of the relationship between depressed haem synthesis and altered drug metabolism, there is no unequivocal evidence that such a relationship exists.

This study investigates in rats the effects of lead on microsomal cytochrome P-450 and the associated mixed function oxidase system, along with the effects of lead on haem metabolism. The latter include the measurement of the activities of six of the enzymes

of haem biosynthesis including the rate-limiting enzyme ALA synthase and also the measurement of haem oxygenase activity. This latter catabolic haem enzyme has been shown to be activated in hepatic microsomal preparations of lead-poisoned rats (Maines & Kappas, 1976a).

Methods

Materials

Enzymes and co-enzymes were obtained from the Boehringer Corporation (London) Ltd. Chemicals and substrates were obtained from British Drug Houses.

Animal treatment

Male Wistar rats weighing 160 g were injected daily intraperitoneally with lead (as acetate) 10 μmol/kg body weight in saline (0.15 M). Control animals were injected with a similar volume of saline. Animals were pretreated as described above in four groups of 6 lead-treated animals and four groups of 6 control animals. They received one daily injection for 1, 3, 7 or 14 days and were killed 24 h after the last injection. The animals were deprived of food for 18 h before they were killed but received water *ad libitum*.

Preparation of liver microsomes

The animals were killed by cervical dislocation and their livers perfused *in situ* with ice cold saline; all further procedures were carried out at 4°C. The livers were quickly removed, blotted and weighed and 25% (w/v) homogenates in 0.15 M KCl were prepared with a Potter-Elvehjem homogenizer. The homogenate was spun for 10 min at 900 *g* and supernatant produced spun at 20,000 *g* for 15 minutes. The microsome containing supernatant was spun at 105,000 *g*, the microsomal pellet produced was washed by resuspending it in 0.15 M KCl and recentrifuging for a further 60 min at 105,000 *g*. The resulting washed microsomal pellet was suspended in 0.15 M KCl to give a concentration of 6 mg protein/ml.

Cytochrome P-450. The quantity of microsomal cytochrome P-450 was determined by the method of Omura & Sato (1964). The amount of cytochrome P-450 was expressed as nmol of P-450/mg microsomal protein.

Cytochrome b_5. The quantity of microsomal cytochrome b_5 was determined by the method of Omura & Sato (1964). The amount of cytochrome b_5 was expressed as ΔE423 nm–410 nm/mg microsomal protein.

Aniline hydroxylase. Microsomal aniline hydroxylase activity was determined by the method of Schenkman, Remmer & Eastbrook (1967). The activity was expressed as nmol *p*-amino phenol produced per mg microsomal protein in 1 h (nmol *p*-aminophenol.mg^{-1} microsomal protein.h^{-1}) at 37°C.

Aminopyrine demethylase. Microsomal aminopyrine demethylase activity was determined by the method of Cochin & Axelrod (1959). The activity was expressed as nmol of formaldehyde formed per mg microsomal protein in 1 h (nmol formaldehyde.mg^{-1} microsomal protein.h^{-1}) at 37°C.

Haem oxygenase. Microsomal haem oxygenase activity was determined by the method of Maines & Kappas (1975). The activity was expressed as nmol bilirubin formed per mg microsomal protein in 1 h (nmol bilirubin.mg^{-1} microsomal protein.h^{-1}) at 37°C.

Haem biosynthetic enzymes

The activity of haem biosynthetic enzymes ALA synthase, ALA dehydratase, porphobilinogen (PBG) deaminase, uroporphyrinogen (URO) decarboxylase, coproporphyrinogen (COPRO) oxidase and ferrochelatase were determined by the method of Brodie, Thompson, Moore, Beattie & Goldberg (1977). ALA synthase activity was determined in 50% (w/v) homogenates in 0.15 M KCl from whole liver, in the 6 animals in each group. The remaining four haem biosynthetic enzyme activities were determined in 33% (w/v) homogenates in 0.15 M KCl from pooled liver samples, each sample being from two animals in the appropriate group.

The activities were expressed as: ALA synthase, nmol ALA formed per g homogenate protein in 1 h at 37°C; ALA dehydratase, μmol ALA utilised per g homogenate protein in 1 h at 37°C; porphobilinogen deaminase, nmol URO formed per g homogenate protein in 1 h at 37°C; uroporphyrinogen decarboxylase, nmol COPRO formed per g homogenate protein in 1 h at 37°C; coproporphyrinogen oxidase, nmol PROTO formed per g homogenate protein in 1 h at 37°C; ferrochelatase % ct/min of ^{59}Fe incorporated into haem per g protein in 1 hour.

Lead levels

Lead levels were determined in microsomal suspensions and liver homogenates by flameless atomic absorption spectrophotometry (Perkin Elmer 306 with HGA 72 attachment) by the method of Meredith, Moore & Goldberg (1977).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Results

Effect of lead pretreatment on liver weight, homogenate and microsomal protein content and homogenate and microsomal lead levels

The results in Table 1 show that there was no significant difference in liver weight between control and lead-treated animals in any of the four groups. There was no significant difference in homogenate protein content between control and lead-treated animals in any of the four groups; this was also true of microsomal protein content. Liver homogenate lead levels showed a progressive increase with increase in lead treatment (Table 1), in each group the lead levels being significantly higher ($P < 0.001$) in the lead-treated animals in comparison to the appropriate control animals. There was a similar progressive rise in microsomal lead levels in the lead-treated animals as compared to the appropriate controls, although this was not statistically significant in the first group of animals.

Effect of lead pretreatment on the activities of ALA synthase, microsomal mixed function oxidase enzymes and haem oxygenase

With progressive increase in lead treatment there was an associated rise in the activity of hepatic ALA synthase in the lead-treated animals as compared to their appropriate controls (Table 2). This was highly significant ($P < 0.001$) in the lead-treated animals of groups 3 and 4 with respect to their appropriate controls. Increase in the activity of ALA synthase was associated with decreased microsomal cytochrome P-450 content, increasing lead pretreatment resulting in progressively lower cytochrome P-450 content. This depression was significant in the lead-treated animals of group 2 ($P < 0.05$), group 3 ($P < 0.001$) and group 4 ($P < 0.001$) with respect to their appropriate controls (Table 2).

A least squares regression analysis of the inverse relationship between ALA synthase and cytochrome P-450 reveals a highly significant relation ($r = 0.76$, $P < 0.001$) of the form:

$$\text{cytochrome P-450} = [41.4 \pm 3.4]/\text{ALA synthase} + [0.098 \pm 0.034] \text{ (Figure 1).}$$

Although, like the cytochrome P-450 content, cytochrome b_5 content was decreased with increasing lead pretreatment, the effect was not so marked and only attained statistical significance ($P < 0.05$) in the lead-treated animals of groups 3 and 4 (Table 2).

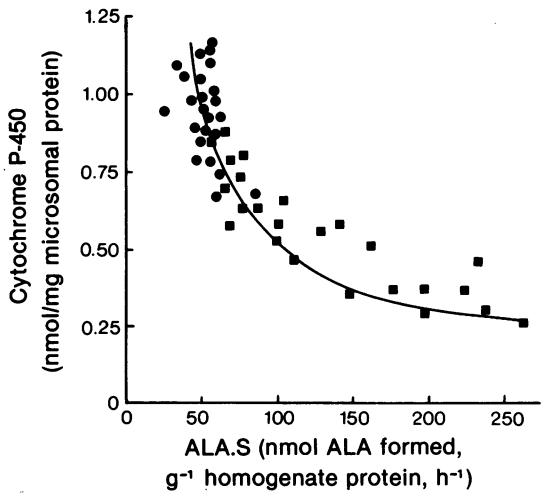

Figure 1 Correlation of hepatic δ-aminolaevulinic acid (ALA) synthase activity with microsomal cytochrome P-450 levels in control (●) and lead-treated rats (■).

The activities of the two cytochrome P-450-dependent microsomal enzymes, aniline hydroxylase and aminopyrine demethylase, followed a similar pattern to that observed for cytochrome P-450 content. Increasing pretreatment with lead resulted in progressively decreased activity of the enzymes when compared to appropriate controls (Table 2).

The activity of the haem degradative enzyme, haem oxygenase, was increased by pretreatment with intraperitoneal lead. This was statistically significant in the lead-treated animals of groups 3 ($P < 0.05$) and 4 ($P < 0.005$) when compared to their controls.

Effect of lead pretreatment on the activities of haem biosynthetic enzymes

The pretreatment of the animals with lead resulted in raised hepatic levels of lead as determined in hepatic homogenates, increasing lead pretreatment resulting in progressively increasing homogenate lead levels (Table 3). Associated with this increase in homogenate lead levels was a change in activity of various enzymes of haem biosynthesis. As has already been noted, lead pretreatment resulted in a significant increase in the activity of the rate-limiting enzyme of haem biosynthesis, ALA synthase (Table 2). Increased hepatic lead levels were associated with decreased activities of the enzymes, ALA dehydratase, COPRO oxidase and ferrochelatase (Table 3). Statistical analysis of these observations was not carried out because of the small number of samples in each group.

Table 1 Effect of lead treatment on liver weight, homogenate and microsomal protein content and homogenate and microsomal lead levels in rats

Group	Treatment (i.p. daily dose/ kg body wt)	Liver wt (g)	Liver homogenate protein content (mg/g wt liver)	Microsomal protein content (mg/g wt liver)	Liver homogenate lead (nmol/g protein)	Microsomal lead (nmol/g protein)
Control (1)	Saline	6.2 ± 1.3	180 ± 20	12.5 ± 1.7	18.8 ± 5.2	66.4 ± 18.2
Lead (1)	1 × 10 μmol	5.9 ± 1.6	172 ± 21	12.0 ± 2.0	167 ± 13.4*	82.3 ± 12.4
Control (2)	Saline	6.2 ± 1.2	188 ± 19	13.0 ± 1.8	16.9 ± 4.7	80.2 ± 9.2
Lead (2)	3 × 10 μmol	6.0 ± 1.5	195 ± 25	13.5 ± 1.9	338 ± 28.4*	301 ± 22.1*
Control (3)	Saline	6.0 ± 1.7	182 ± 20	13.7 ± 2.1	16.2 ± 3.9	68.8 ± 8.7
Lead (3)	7 × 10 μmol	5.8 ± 1.8	169 ± 18	14.8 ± 2.5	1151 ± 124*	808 ± 64.2*
Control (4)	Saline	6.0 ± 1.7	181 ± 15	14.9 ± 2.8	18.4 ± 5.8	73.1 ± 15.2
Lead (4)	14 × 10 μmol	5.3 ± 1.8	170 ± 22	12.1 ± 2.6	1864 ± 18.1*	1230 ± 101*

Each value is the mean result from 6 animals ± s.d.
* $P < 0.001$ with respect to appropriate control.

Table 2 Effect of lead treatment on the activities of δ-aminolaevulinic acid (ALA) synthase, microsomal fixed function oxidase enzymes and haem oxygenase in rats

Group	ALA synthase (nmol ALA . g^{-1} homogenate protein . h^{-1}	Cytochrome P-450 (nmol cP450/ mg microsomal protein)	Cytochrome b_5 (Δ E423-410/mg microsomal protein)	Aniline hydroxylase (nmol p-amino phenol . mg^{-1} microsomal protein . h^{-1})	Aminopyrine demethylase (nmol HCHO . mg^{-1} microsomal protein . h^{-1})	Haem oxygenase nmol bilirubin . mg^{-1} microsomal protein . h^{-1})
Control (1)	48.1 ± 12.2	0.82 ± 0.09	0.063 ± 0.013	43.5 ± 6.5	222 ± 28	1.25 ± 0.28
Lead (1)	56.5 ± 10.1	0.76 ± 0.12	0.066 ± 0.012	41.6 ± 6.7	198 ± 18	1.38 ± 0.26
Control (2)	51.8 ± 15.2	0.92 ± 0.22	0.062 ± 0.016	43.6 ± 6.9	208 ± 35	1.25 ± 0.19
Lead (2)	62.5 ± 9.8	0.64 ± 0.07†	0.056 ± 0.006	29.1 ± 6.2†	154 ± 29†	1.36 ± 0.31
Control (3)	56.0 ± 15.5	0.83 ± 0.06	0.066 ± 0.007	39.1 ± 5.4	211 ± 26	1.20 ± 0.23
Lead (3)	137.4 ± 21.4‡	0.52 ± 0.05‡	0.058 ± 0.003†	27.4 ± 5.6†	138 ± 19*	1.62 ± 0.35†
Control (4)	62.5 ± 14.2	0.94 ± 0.23	0.065 ± 0.006	39.6 ± 6.5	217 ± 24	1.31 ± 0.22
Lead (4)	194 ± 33.5‡	0.39 ± 0.13‡	0.053 ± 0.010†	21.7 ± 3.2‡	105 ± 18‡	1.86 ± 0.18*

Animals of each group were treated as indicated in Table 1; each figure is the mean result from six animals ± s.d.
† $P < 0.05$; * $P < 0.005$; ‡ $P < 0.001$.

Table 3 Effect of lead on the activities of haem biosynthetic enzymes in rats

Group	*ALA dehydratase* (*μmol ALA . g^{-1} protein . h^{-1}*)	*PBG deaminase* (*nmol URO . g^{-1} protein . h^{-1}*)	*URO decarboxylase* (*nmol COPRO . g^{-1} protein . h^{-1}*)	*COPRO oxidase* (*nmol PROTO . g^{-1} protein . h^{-1}*)	*Ferrochelatase* (*ct/min ^{59}Fe incorp. into haem . g^{-1} protein . h^{-1}*)	*Homogenate lead* (*nmol Pb/ g protein*)
Control (1)	20.2 (17.6–24.8)	48.9 (41.2–54.4)	18.9 (18.4–19.8)	34.1 (30.1–38.2)	2964 (2766–3272)	16.4 (13.8–19.9)
Lead (1)	9.8 (8.7–10.9)	48.8 (43.2–56.4)	17.9 (16.7–18.6)	39.3 (38.2–40.8)	2841 (2729–3063)	159 (142–173)
Control (2)	19.8 (19.2–20.4)	46.2 (45.0–47.5)	18.4 (16.3–20.0)	33.4 (30.0–36.1)	3664 (3408–3976)	19.3 (16.2–24.4)
Lead (2)	9.9 (9.6–10.8)	48.3 (46.0–51)	19.2 (18.0–24.0)	29.2 (26.5–33.0)	2812 (2698–2982)	329 (290–366)
Control (3)	17.1 (16.8–17.9)	48.9 (41.0–54.0)	16.9 (15.1–18.6)	35.2 (28.4–42.4)	3616 (3432–3681)	18.9 (16.2–20.3)
Lead (3)	5.4 (5.1–5.8)	49.2 (45.0–53.0)	15.8 (14.1–17.8)	11.9 (10.6–14.4)	2398 (2200–2541)	1043 (890–1432)
Control (4)	18.2 (15.6–20.8)	42.8 (39.2–43.1)	17.2 (13.0–21.6)	38.2 (25.8–46.1)	3341 (2860–3640)	19.3 (14.6–21.3)
Lead (4)	3.3 (2.5–3.7)	43.4 (41.1–45.0)	15.6 (14.2–16.7)	14.8 (14.2–16.2)	1963 (1755–2184)	1682 (1242–1926)

Animals of each group were treated as indicated in Table 1 but each measurement was made on pooled liver samples from two animals which had been treated identically. Results given are the means of three observations with the range in parentheses below.

Effect on enzyme activities of in vitro *additions of lead to rat hepatic microsome preparations*

Microsomal preparations from control male Wistar rats were prepared and the parameters of the mixed function oxidase system and haem oxygenase were measured following the *in vitro* addition of lead, to raise the microsomal lead concentration from 80 to 280, 580, 1080, 5080 or 50,080 nmol lead/g microsomal protein.

Cytochromes P-450 and b_5 content were unaffected by *in vitro* additions of lead whilst the activities of the enzymes, aniline hydroxylase and aminopyrine demethylase, were only decreased at the highest concentration of lead. The activity of haem oxygenase was not increased by *in vitro* additions of lead and in fact was slightly inhibited by the highest concentration of lead.

Discussion

Results of previous animal (Alvares *et al.*, 1972; Scoppa *et al.*, 1973) and human (Alvares *et al.*, 1975; Meredith *et al.*, 1977) studies have demonstrated that lead inhibits the elimination of drugs metabolized by the cytochrome P-450-dependent microsomal mixed function oxidase system. It has been suggested by these authors that the decreased capacity for drug handling results from reduced availability of cytochrome P-450 due to inhibition of haem biosynthesis by lead. Indirect evidence of such a mechanism has been obtained by measuring inhibition of the activity of the haem biosynthetic enzyme ALA dehydratase (Alvares *et al.*, 1972; Scoppa *et al.*, 1973; Meredith *et al.*, 1977). It has also been suggested that lead exerts its action directly on the hepatic mixed function oxidase system (Alvares *et al.*, 1972).

Results from the present study provide unequivocal evidence that lead administration to rats results in decreased cytochrome P-450 content and inhibition of *in vitro* N-demethylase and hydroxylase activities and that this is associated with a depression by lead of haem biosynthesis. Increasing lead administration resulted in a progressive decrease in cytochrome P-450 content and a progressive decrease in the activities of aniline hydroxylase and aminopyrine demethylase. As has been noted previously (Maxwell & Meyer, 1976), associated with this impairment of the mixed function oxidase system there was an increase in the activity of the enzyme ALA synthase. This mitochondrial enzyme catalyses the initial step of the haem biosynthetic pathway and is the decisive controlling factor for overall rate of haem synthesis in the liver (Marver & Schmid, 1972). The activity of this enzyme appears to be regulated by the end product of the pathway, haem, and for this reason a regulatory 'pool' of hepatic haem has been postulated (De Matteis, 1971; Tschudy & Bonkowsky, 1972; Meyer & Schmid, 1973; Watson, 1975). The precise mechanisms of this regulation remains controversial (Scholnick, Hammaker & Marver, 1969; Sassa & Granick, 1970; Hayashi, Kurashima & Kikuchi, 1972), although evidence for its existence has come from various studies. When haem was administered to rats (Marver, Schmid & Schutzel, 1968; Watson, 1975), or added to the incubation medium of cultured chick embryo liver cells (Granick, 1966; Sassa & Granick, 1970; Strand, Manning & Marver, 1972), it blocks the response of ALA synthase to inducing chemicals such as phenobarbitone or allyl isopropylacetamide.

The inverse relationship between ALA synthase and cytochrome P-450 content noted in the present study (Figure 1) provides further evidence of the control mechanism whereby a decrease in the regulatory 'pool' of haem, as assessed by the microsomal content of the haemoprotein cytochrome P-450, results in an increase in the activity of ALA synthase. The decrease in the microsomal content of cytochrome P-450 associated with lead pretreatment is possibly due to the observed decreases in the activities of the haem biosynthetic enzymes ALA dehydratase, COPRO oxidase and ferrochelatase reducing the available 'free haem' for cytochrome P-450 synthesis. It is interesting to note that these results obtained from the livers of rats treated with lead follow a similar pattern to that observed in the peripheral blood of a group of lead workers (Campbell, Brodie, Thompson, Meredith, Moore & Goldberg, 1977).

No direct action of lead on the hepatic mixed function oxidase system is evident from the effects of lead added *in vitro* to microsomal preparations, lead only exerting an inhibitory effect at very high concentrations that are incompatible with life.

The activity of the microsomal haem degradative enzyme, haem oxygenase was found to be increased as the pretreatment of the animals with lead increased. This is in agreement with the findings of Maines & Kappas (1976a) but conflicts with the model suggested by Bissell & Hammaker (1976). Their model considered that ALA synthase and haem oxygenase are inter-related with respect to their regulation by haem. They further predicted that these enzyme activities vary reciprocally, this theory having experimental support in the studies of Schacter (1975). From this theory one would expect depletion of the regulatory haem 'pool' which would activate ALA synthase but depress haem oxygenase. In the present study the experimental evidence does not support this theory, for as lead pretreatment of the rats inhibits hepatic haem synthesis and thereby depletes the regulatory haem 'pool' there is not only an associated

increase in the activity of ALA synthase but also a rise in the activity of haem oxygenase.

The results of the present study are consistent, however, with the theory postulated by Maines & Kappas (1976b) that the mode of action of metals in inducing haem oxygenase is based on a repressor component in the regulatory mechanism of haem oxygenase. They suggested that this repressor has an –SH active constituent, the oxidation-reduction capacity of which is necessary for controlling haem oxygenase production. If the oxidation-reduction cycle of the –SH groups of this component is blocked, as would occur after treatment with lead which has a high affinity for –SH groups, the regulatory function of this cellular constituent on haem oxygenase would be lost. The system would then function without repressive regulation, leading to an exaggerated synthesis of the enzyme.

Thus, the impairment of the mixed function oxidase system in lead intoxication has been shown to be associated with a decrease in some of the enzymes of haem biosynthesis and an increase in the haem degradative enzyme haem oxygenase. The present work does not allow any conclusion to be reached on the relative importance of inhibition of haem synthesis and of accelerated haem degradation in the reduction of microsomal cytochrome P-450 content following lead administration. Both mechanisms are possible and are not mutually exclusive.

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