in cytosol of rat liver [12]. The induction of the enzyme by phenobarbital injection plays an important role in acetylation process in drug metabolism.

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Malathion A and B esterases of mouse liver—II Effect of EPN *in vitro* and *in vivo*

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EPN [*O*-ethyl *O*-*p*-nitrophenyl phenylphosphorothionate] potentiates the toxicity of malathion [*S*-(1,2-dicarbethoxyethyl) *O,O*-dimethyl phosphorodithioate] in mammals [1]. This synergism is attributed to the inhibition by EPN, via its oxygen analogue EPNO formed by oxidative desulphation in the liver, of non-specific carboxylesterases which normally detoxify malathion [2, 3]. Malaoxon, the active oxygen analogue of malathion would thus accumulate resulting in increased toxicity. Although the above sequence of events is a satisfactory working hypothesis to explain EPN–malathion synergism, some anomalies have been pointed out [4–6]. More recently, another inconsistency has been reported by Cohen and Murphy [7]. Whereas one would expect that the ability of tissues to hydrolyse malathion would be considerably reduced by the administration of EPN, the inhibition of this activity seems to be the lowest. Cohen and Murphy found that the inhibition of the esterase activities in mouse liver with diethyl succinate, methyl butyrate and triacetin as substrates was 90, 82 and 70%, respectively, while that with malathion itself was only 30%.

In a previous communication [8] we reported that there are two types of esterases in mouse liver which hydrolyse malathion. We had named these malathion A and B esterases. The effect of EPN *in vitro* and *in vivo* on these esterases is reported in this communication.

MATERIALS AND METHODS

The materials and methods used were the same as in the previous paper [8]. For injections of EPN, the required amount/kg was dissolved in 0·4 ml ethanol and diluted to 4 ml with propylene glycol. Appropriate volumes were injected intraperitoneally into mice and the animals were sacrificed after 1 hr. Malathion A and B esterase activities were determined as described in the previous communication. Esterase activity with phenyl acetate (PA) as substrate was determined at 20° according to the method given by Ramachandran and Ågren [9].

RESULTS

In vitro EPN at 10^{-5} M had only a weak inhibitory effect on malathion A and B esterases, the extent of inhibition being 17 and 13% respectively. EPNO had no effect

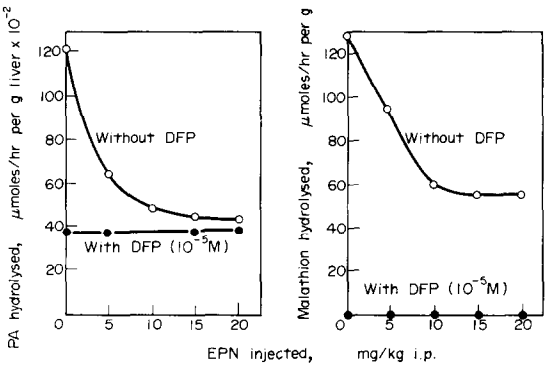

Fig. 1. Effect of injected EPN on mouse liver esterase activity with phenyl acetate (*left*) and malathion (*right*) as substrates. Conditions: for phenyl acetate, 0·067 M phosphate buffer pH 7·2 with 0·1% Triton X-100, phenyl acetate 10^{-2} M, 20°; for malathion B esterase 0·05 M Tris–HCl buffer pH 7·6 with 0·1% Triton X-100, malathion 10^{-3} M, 37°. In both cases duplicates were run simultaneously with homogenates which had been preincubated with 10^{-5} M DFP for 30 min at 0°. Each point is the average of 5–10 separate experiments. The residual malathion B esterase activity was completely inhibited by DFP (see the line at the base of figure on right).

Table 1. Effect of EPN *in vivo* on malathion a esterase activity of mouse liver

Injection	A esterase (μmoles malathion hydrolysed/ g of liver per hr)	P
1. None	26·0 ± 0·94 (12)†	
2. EPN 15 mg/kg/1 hr	31·8 ± 0·83 (20)	0·0005 (1)‡
3. EPN 15 mg/kg/5 hr	31·2 ± 2·45 (7)	0·0005 (1) 0·25 (2)
4. Hydrolysed EPN 15 mg/kg/1 hr	32·7 ± 1·32 (14)	0·0005 (1)
5. PNP 6·5 mg/kg/1 hr*	26·5 ± 0·59 (7)	0·20 (1)
6. Ethanol 10% + propylene glycol 90%/1 hr	26·3 ± 1·54 (6)	0·35 (1)

* Molar equivalent of 15 mg of EPN.

† Values are mean ± S.E.M. with the number of determinations in parenthesis.

‡ Number of group used for comparison is given in parenthesis.

on the A esterase. As mentioned earlier EPNO had a strong inhibitory effect on the B esterase.

When EPN was injected into mice at dose levels from 5 mg to 10 mg/kg, the malathion B esterase activity was found to decrease steeply from 128 ± 3(8) μmoles/g wet liver per hr to 59 ± 3(5) μmoles/g per hr at 10 mg/kg, the inhibition being about 54% at this level (see Fig. 1, right). Standard deviations and number of experiments (in parentheses) are given. On increasing the dose to 15 and 20 mg/kg there was no appreciable further decrease in the malathion B esterase activity, the value not falling below 55 ± 3(10) μmoles/g per hr. This represents an inhibition of 59%. When these homogenates were incubated with 10^{-5} M DFP for 30 min the residual malathion B esterase activity was almost completely inhibited (see curve at base line on the right). In a few experiments higher doses of 30 and 40 mg/kg were injected into mice which had been protected with 17·4 mg/kg of atropine sulphate (Merck) and 35 mg/kg of toxogonin [bis-(4-hydroxyiminomethyl pyridinium) ether dichloride] (Merck), in combination, injected intraperitoneally, 15 min before EPN. It is known that these antidotes do not prevent the incorporation of organophosphates in non-specific carboxylesterases [10, 11]. In these cases also the malathion B esterase activity remained at the same level, and the residual activity could be completely inhibited by 10^{-5} M DFP. Paraoxon as well as EPNO also completely inhibited the residual B esterases *in vitro* at 10^{-5} M. To determine whether EPN *per se* effected only a partial inhibition of B esterases *in vivo*., esterase activity was determined using phenyl acetate as the substrate. The relationship between EPN injected and the enzyme activity with this substrate is given in Fig. 1 (left). The initial activity of 12452 ± 278(8) μmoles of PA/g liver per hr decreased to 4764 ± 717(9) at 10 mg/kg of EPN. The activity then levelled off at about 4200 μmoles when the EPN dose was raised to 15 and 20 mg/kg. But in contrast to experiments where malathion was used as substrate, this residual PA esterase activity was not appreciably further decreased by DFP. In fact, at these levels of EPN, the activity with and without DFP seems to be about the same. If B esterase activity is taken as the difference between the total PA hydrolysed, and that in the presence of DFP, it can be shown that there is a 70% inhibition of this activity at 5 mg/kg EPN, and 95% inhibition at 10 mg/kg EPN. On increasing the dose of EPN there is no further reduction in the B esterase activity. Thus the picture is clearer with PA as the substrate. The B esterase activity is almost completely inhibited by injected EPN. With malathion as the substrate only a part of the B esterase activity is inhibited by injected EPN, the remaining part being further inhibited by DFP, paraoxon and EPNO *in vitro*.

The effect of injected EPN on malathion A esterase was then studied. In this case, instead of administering EPN in increasing doses, experiments were done at only one level, viz. 15 mg/kg. Controls without EPN were run simultaneously. In all cases the homogenates were preincubated with DFP and GSH and the buffers contained as usual 10^{-3} M 2-mercaptoethanol. The malathion A esterase activities are given in Table 1. The values of groups 1 and 2 represent the malathion A esterase activity without and with EPN respectively, the sacrifice time being 1 hr. It is seen that the A esterase activity is increased in EPN-treated animals. The difference was highly significant ($P < 0.0005$). When the animals were sacrificed after 5 hr the value remained practically the same as that at 1 hr. The P between groups. 1 and 3 was < 0·0005 and that between 2 and 3 was > 0·25. Thus the interval between injection and sacrifice does not seem to have any significant effect.

Since malathion A esterase is activated by SH containing reagents, and EPN on hydrolysis will give an SH containing compound, viz. *o*-ethyl phenyl phosphonothioate [12], a sample of EPN which had been hydrolysed by NaOH was administered. For this, 15 mg of EPN was dissolved in 0·5 ml of ethanol and 1 ml of 0·5 N NaOH was added. After standing for 3 hr at room temperature, 1 ml of 0·5 N HCl was added to exactly neutralise the NaOH. The half-life of EPN in ethanol–NaOH is only about 8 min [13]. The solution was then diluted to 4 ml and administered to 14 mice (2 sets of 7 each) at 4 ml/kg. The malathion A esterase activity was found to be higher than that of the controls (group 4). Trials were run with only *p*-nitrophenol (PNP) in equimolar concentration to 15 mg/kg of EPN. In this case the value did not differ significantly from that of the controls (group 5). Likewise, the medium used (10% ethanol + 90% propylene glycol) did not have any effect on malathion A esterase activity (group 6). Thus, only EPN and its P and S containing degradation product seem to enhance the activity of malathion A esterase *in vivo*. In all the above experiments it was found necessary to include reduced glutathione or other SH activators in the assay system without which the malathion A esterase activity was not detectable.

DISCUSSION

EPN injected into mice acts differently and in opposite directions on malathion A and B esterases. The A esterase activity is enhanced while the B esterase activity is partially inhibited.

Three components of organophosphate sensitive (B-esterase) activity could be recognized: (1) the activity on phenyl acetate which is inhibited by injected EPN almost to the same extent as DFP *in vitro* (the space between the two curves in Fig. 1, left, for activity with and without DFP is quite narrow), (2) a component acting on malathion at the carboxyethyl ester group which is inhibited by EPN *in vivo*, and also by DFP, paraoxon and EPNO *in vitro*. (Fig. 1, right), and (3) a component acting on malathion at the carboxyethyl ester group which is not inhibited by injected EPN but is completely inhibited by DFP, paraoxon and EPNO *in vitro* (the wide space between the curve and the base line in Fig. 1, right). The components (1) and (2) may or may not be identical, but (3) seems to be different in not being sensitive to injected EPN. There is also a possibility that this may not be a different enzyme but only a part of (2) which is located in a position in the intact animal cell which is not accessible to EPN and its active component EPNO *in vivo*, but on homogenization is exposed and acted upon by EPNO *in vitro*.

Malathion A esterase activity is not only not inhibited but is significantly increased in EPN-treated mice. Hydrolysed EPN also increases the A esterase activity but PNP does not do so. An explanation for these findings is not possible at this stage. However, the increase in the malathion A esterase activity in EPN-treated animals, may to some extent, explain one anomaly, viz. the poor over-all inhibition of malathion degrading activity in the presence of EPN *in vivo*.

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Hexachlorobenzene-induced porphyria in rats—Relationship between porphyrin excretion and induction of drug metabolizing liver enzymes

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The porphyrogenic action of the fungicide hexachlorobenzene (HCB) in man as in some types of laboratory animals has been well known for several years [1, 2].

The mechanism by which HCB induces the porphyria is not known. Repeated administration of the compound to rats causes a marked increase of the urinary porphyrin-precursors, 5-aminolaevulinic acid (ALA) and porphobilinogen (PBG) between the sixth and tenth week of treatment. The excretion of both of these precursors by humans or rabbits is not increased by HCB, but all three species excrete increased amounts of uro- and coproporphyrin in their urine. Three or four weeks after starting the experiments the neurotoxic effects of HCB including clonic convulsions, tremor, hyperexcitability, and changes in hair color become obvious. Depending on the duration of the HCB administration some of the animals die after showing signs of general weakness.

Only limited information is available about the metabolism of HCB. Parke and Williams [3] could not detect any metabolites of HCB in the urine or expired air. No glucuronidated derivatives, ethereal sulfate or mercapturic acid were excreted.

In the present experiments, a search has been made for relationships between the onset of the experimental porphyria and the changes in the activity of liver drug metabolizing enzymes. ALA and porphyrin levels were estimated in 24-hr urine specimens of 60 male rats fed with an HCB containing diet. Each week, two or three animals were sacrificed and the concentration of cytochrome P-450, microsomal protein and triglycerides and the activity of aniline hydroxylase were determined in the liver. Each week one animal of the control group was investigated in the same way as the rats of the HCB group.

MATERIALS AND METHODS

Male Wistar rats weighing approximately 200 g were fed with a standard diet containing 0·2% HCB (a control group of 15 animals was fed with the same diet containing no HCB). Twenty-four hr before being sacrificed the animals were kept in metabolic cages, during which period

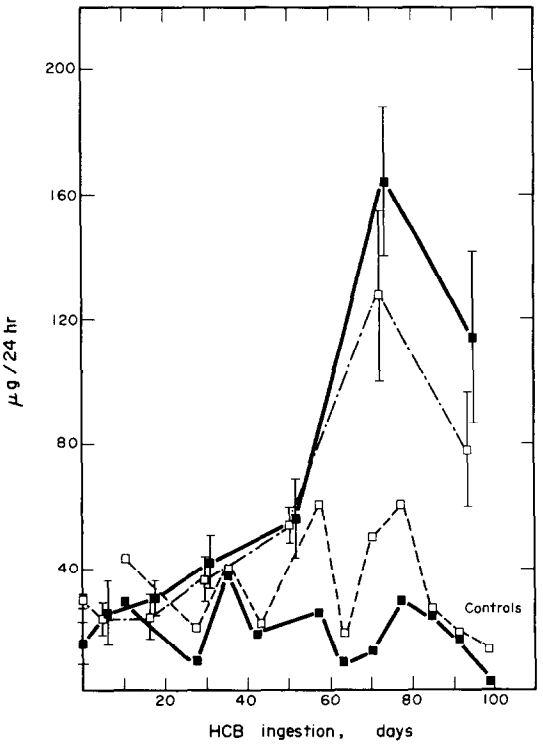

Fig. 1. Excretion pattern of urinary ALA (—□—, μg ALA/24 hr) and porphyrins (—■—, μg porphyrins/24 hr) in rats during long term exposure to HCB. Results are given as means ± S_x (vertical bars) of at least five observations.