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SHORT COMMUNICATIONS

Activation of some organophosphorus insecticides by liver microsomes from phenobarbital-treated mice

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MANY drugs are metabolized by isolated mammalian liver microsomes, in enzyme systems requiring NADPH (or to a limited extent NADH) as well as molecular oxygen. Generally, these reactions result in the formation of pharmacologically inert products. Factors such as age, sex, and nutritional state of the animal influence the activity of these hepatic systems with inverse correlation usually obtained between microsomal activity *in vitro* and the extent of drug action *in vivo*. Drug metabolism has been the subject of a number of recent reviews.[1–3]

It is now well established experimentally that certain organophosphorus insecticides are inefficient anticholinesterases in their original molecular form. When these substances are given to mammals they are oxidized ("activated") to form potent anticholinesterases which are the pharmacologically active compounds. The oxidizing system is remarkably similar to the one responsible for drug metabolism, since it requires the same cofactors (although NADH is in this case as effective as NADPH[4]), it is sensitive to the same inhibitors, and it is located in liver microsomes. The reaction product in the case of phosphorothionates is the corresponding phosphate,[4] while a phosphoramidate like octamethylpyrophosphoramide (schradan) probably gives rise to its hydroxylalkyl derivative.[4, 5]

Increases in microsomal drug-metabolizing enzymes can be brought about by pretreatment of the experimental animals with one of a number of drugs. The long-acting barbiturate, phenobarbital, is one of the substances most commonly used for this purpose. Indirect experimental evidence suggests that the increase in activity resulting from daily injections of phenobarbital is due to enzyme induction.[6–8] Phenobarbital treatment of rats is also accompanied by distinct anatomical changes. There is an increase in liver weight and a proliferation of the endoplasmic reticulum.[9]

Kato[10] has observed an increased toxicity of octamethyl pyrophosphoramide toward female rats pretreated with phenaglycodol or thiopental, while Welch and Coon[11] have reported substantial decrease in the toxicity of a number of organophosphorus insecticides as a result of pretreatment of mice with chlorcyclizine, cyclizine, or phenobarbital.

The purpose of the experimental work in this paper was to investigate the effects of phenobarbital treatment *in vivo* on the ability of isolated mouse liver microsomes to activate certain organophosphate insecticides.

MATERIALS AND METHODS

NADH was purchased from the Sigma Chemical Co. Parathion (99·7% pure) and malathion (99·4% pure) were gifts of the American Cyanamid Co. Schradan was a Monsanto Co. sample extensively purified before use by Dr. E. Y. Spencer of this institute. Malaoxon (chromatographically pure) was a gift from Dr. R. D. O'Brien of Cornell University. Sodium phenobarbital was purchased from British Drug Houses Ltd. Purified bovine erythrocyte acetyl cholinesterase (stabilized with gelatin) was obtained from Nutritional Biochemicals Corp. and the Sigma Chemical Co.

Chemical oxidation of schradan. One g schradan was added to 100 ml phosphate buffer (0·5 M, pH 7·5) and 50 ml 0·2 M $KMnO_4$. The mixture was left to stand at room temperature for approximately 1 hr, at which time the permanganate had largely decolorized. The mixture was then extracted three times with 50-ml volumes of chloroform, the combined extracts extensively dried over Na_2SO_4 and stored at −20°. An appropriate volume of this stock extract was removed immediately prior to the experiment, the chloroform taken off under vacuum, and the residue taken up in water and added to the Warburg flasks.

Preparation of mouse liver microsomes. The mice used were female albinos (20–35 g live weight) supplied by Rolfsmeyer Farms, Madison, Wis. Livers were homogenized in a Potter-Elvehjem unit fitted with a Teflon pestle. Microsomes were prepared and stored as previously described.[12] The pellet was resuspended in 0·25 M sucrose to give a final volume in ml four times the original wet weight of liver in g, and 0·2 ml of this suspension was used in each flask.

Activation. Activation of the organophosphates by microsomes was carried out in phosphate buffer, and the amount of organophosphate oxidized was estimated by a subsequent manometric anticholinesterase assay. The particulars of the reaction were as follows. Initial incubation was at room temperature for 1 hr in open Warburg flasks gently agitated on a wrist-action shaker. The mixture contained 0·02 M phosphate buffer (pH 7·5), 0·03 M $MgCl_2$, $16·6 \times 10^{-3}$ M NADH, and organophosphate and cholinesterase (100–130 μg, dependent on the activity of the particular batch of enzyme used). The concentration of NADH used was in excess of the amount needed for maximal activation in our system. The cholinesterase (ChE) was dissolved in an appropriate volume of water immediately before each experiment. The reaction was started by the addition of microsomes, bringing the total volume to 2·5 ml. At the end of this incubation 0·3 ml of 2·5% $NaHCO_3$ was added to the main compartment and 0·2 ml of 5% acetylcholine bromide to the side arm of the vessel. The flasks were then mounted to the manometers and placed in a Warburg bath at 25°. They were gassed for 10 min with a mixture of 95% N_2 plus 5% CO_2 and equilibrated for an additional 15 min. At 90 min after the addition of microsomes, the acetylcholine was tipped into the main compartment, and its hydrolysis was followed by taking readings at 10-min intervals for 30 min. Per cent inhibitions were calculated on the basis of appropriate controls. Figures given in all tables are the average of three to five determinations with variation between individual values of no more than 5 per cent.

Injections. Mice were injected i.p. with five daily doses of 40 mg phenobarbital/kg body weight in 0·9% NaCl. Control animals received the same number of 0·9% NaCl injections. Animals were killed 24 hr after the last injection, and liver microsomes were prepared as outlined above. The final microsomal suspension was standardized by minor volume adjustment to a constant turbidity measurement in a Klett colorimeter.

RESULTS AND DISCUSSION

Three organophosphorus compounds—schradan, malathion, and parathion—were incubated with microsomes from control and phenobarbital-injected mice under conditions necessary for activation of these compounds. The extent of the activation reaction was estimated by a cholinesterase activity

TABLE 1. ACTIVATION OF SOME ORGANOPHOSPHATES BY MICROSOMES FROM CONTROL AND PHENOBARBITAL-TREATED MICE*

Organophosphate	Amount added (μmoles)	ChE inhibition (%)	
		Control mice	Treated mice
Schradan	0·442	57	93
	0·221	34	72
	0·110	23	48
	0·044	2	20
Malathion	0·227	45	52
	0·133	39	42
	0·056	18	32
	0·023	9	17
Parathion	$6·8 \times 10^{-4}$	75	56
	$5·2 \times 10^{-4}$	57	35
	$4·3 \times 10^{-4}$	20	28
	$3·4 \times 10^{-4}$	7	12

* Activation procedure and anticholinesterase assay as outlined under methods. Schradan solutions made up in distilled water. Stock solutions of malathion and parathion were prepared in 95% ethanol and appropriate dilutions made for each experiment with distilled water.

assay as explained above. The results of these experiments are given in Table 1. It should be noted that, at the concentrations used, none of the compounds had any anticholinesterase activity when microsomes were omitted. It is evident from Table 1 that microsomes from phenobarbital-treated mice activate schradan at a considerably faster rate than do control microsomes. This is seen with all schradan concentrations used. In the case of malathion, there is a more efficient activation at the two lower concentrations used, but the difference is not nearly so striking as in the case of schradan. There is no evidence of a similar effect with parathion. In fact, with $6{\cdot}8 \times 10^{-4}$ μmoles it seems that less paraoxon is produced by microsomes of treated mice, with a resulting lower per cent inhibition of ChE. The findings on parathion activation in Table 1 do not seem to agree with those of Welch and Coon,[11] who have reported a considerable increase of parathion activation by liver slices from mice pretreated with chlorcyclizine, another microsomal enzyme stimulator.

Any critical evaluation of these results must consider that the amount of anticholinesterase present at the end of the incubation depends upon the rates of two different reactions. Activation on the one hand would increase the anticholinesterase activity of the incubation mixture, while hydrolytic degradation of the active material would have the opposite effect. It was necessary, therefore, to look for any possible differences between microsomes from control and treated mice in their ability to degrade active schradan and malaoxon. In these experiments, we used the product of permanganate oxidation of schradan, which has been shown to be identical with the active anticholinesterase produced by microsomal oxidation of schradan.[5] In order to guard against activation of any unoxidized schradan present in the permangante oxidation product, NADH was omitted from the microsomal system. Cholinesterase assays in this case are a direct measure of the degrading ability of microsomes. The results of these experiments are given in Table 2. It is clear from these figures

TABLE 2. DEGRADATION OF ACTIVE SCHRADAN AND MALAOXON BY MICROSOMES FROM CONTROL AND PHENOBARBITAL-TREATED MICE*

Organophosphate	ChE inhibition (%)		
	No microsomes	Control mice	Treated mice
Active schradan	53	45	27
Malaoxon	86	21	24

* Incubation procedure and anticholinesterase assay as outlined under methods except that NADH was omitted. For the experiments with active schradan, appropriate dilutions of an aliquot of the chloroform extract of the chemically oxidized material were used (see Methods). Malaoxon was used at $1{\cdot}06 \times 10^{-6}$ M final concentration.

that microsomes of treated mice degrade active schradan more efficiently than do control microsomes, while this difference does not seem to exist in the case of malaoxon. In view of these findings, the results in Table 1 indicate that when mice are treated with phenobarbital there is a very considerable increase in the ability of their liver microsomes to activate schradan. This is shown by the greatly increased anticholinesterase activity obtained, despite the concomitant increase in the ability of the same microsomes to degrade the active material (Table 2). In other words, the true increase in the capacity for schradan activation is larger than is apparent from Table 1. In the case of malathion activation, the results of Table 1 may be taken at face value, since the degrading activity of microsomes from treated and untreated mice was the same (Table 2).

It was of interest to find out whether phenobarbital could influence the oxidation of schradan when the barbiturate is added *in vitro* to control microsomes. In this connection, Fenwick *et al.* have reported that barbiturates inhibit the activation of dimefox by liver microsomes.[13] The results (Table 3) show that at high concentrations phenobarbital inhibits the activation of schradan, whereas at 10^{-5} M and lower there is no effect. Evidently the stimulatory action of phenobarbital treatment on the activation of schradan cannot be explained by the possible presence of the unchanged drug in the incubation mixture.

In conclusion, it seems that treatment of mice with phenobarbital results in a considerable increase in the ability of their microsomes to oxidize the phosphoramidate schradan. There is some evidence of a similar but much less striking effect with the phosphorothionate malathion, while there is no evidence of the same effect with parathion, another phosphorothionate.

TABLE 3. THE EFFECT OF PHENOBARBITAL, ADDED *in vitro*, ON THE ACTIVATION OF SCHRADAN BY MOUSE LIVER MICROSOMES*

Phenobarbital concentration (M)	ChE inhibition (%)
0	44
10^{-6}	43
10^{-5}	45
10^{-4}	36
10^{-3}	18

* Activation procedure and anticholinesterase assay as outlined under methods, with 0·33 μmole schradan added per flask. Phenobarbital concentrations given are final in the 3-ml incubation mixture of the cholinesterase assay.

In the past, it seemed troublesome that the activation of different organophosphorus compounds appeared to be mediated by the same microsomal enzyme system, even though these substances often had widely different molecular structures.[4] From the present results it is now more likely that different systems are responsible for the activation of the three compounds studied.

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