Biochemical Pharmacology, Vol. 24, pp. 1221–1225. Pergamon Press, 1975. Printed in Great Britain.

INHIBITION OF HEPATIC DRUG-METABOLIZING ENZYMES BY THIOPHOSPHATE INSECTICIDES AND ITS DRUG TOXICOLOGICAL IMPLICATIONS

MITSURU UCHIYAMA, TAKEMI YOSHIDA, KEIKO HOMMA and TSUTOMU HONGO
Pharmaceutical Institute, Tohoku University, Aobayama, Sendai, Japan

(*Received* 30 *March* 1974; *accepted* 22 *November* 1974)

Abstract—Thiophosphate insecticides—fenitrothion, diazinon and methylparathion—inhibit hepatic drug-metabolizing enzyme activity, which was assayed using aminopyrine and aniline as substrates. About 50 per cent inhibition was noted 4 hr after the injection of 25 mg/kg of fenitrothion into mice. The addition of thiophosphates to a microsomal drug-metabolizing enzyme system *in vitro* also produced an effective inhibition: IC_{50} was *ca* 10^{-5} M for fenitrothion. DDVP and an oxygenated metabolite of fenitrothion did not show any inhibiting effect either *in vitro* or *in vivo*. The inhibition *in vitro* was competitive, while the kinetics of inhibition *in vivo* appeared to be noncompetitive. Drug metabolism by a rat liver preparation was also inhibited by thiophosphate *in vitro*, but treatment of male rats *in vivo* resulted in little inhibition. The responses of female rats were similar to those of mice. The administration of fenitrothion to mice prolonged the hexobarbital sleeping time and suppressed the oxidative metabolism of parathion in liver preparations.

Organophosphorus insecticides are well known as inhibitors of cholinesterase [1]. In addition, they can react with many other enzymes or biological components [2, 3]. However, it has been assumed to date that organophosphorus insecticides do not produce any significant effect at the levels that normally are found in the environment [4]. This assumption is based on the concept that most of the organophosphates are so readily metabolized in animals that they cannot accumulate to an effective concentration in animal tissue even if they are administered repeatedly. Many reports are available showing the rapid disappearance of organophosphates from animal tissue after a single administration by injection or feeding.

However, preliminary experiments [5] in our laboratory demonstrated that the rate of disappearance of fenitrothion given orally varies according to the method of administration. Fenitrothion, 300 mg/kg, dissolved in 0·1 ml of corn oil was administered orally to one group of male mice and the residual concentration of fenitrothion in liver was determined at a certain time after dosing. Another group was given 30 mg/kg of fenitrothion every day for 10 days and the residue of fenitrothion in liver was also determined at the same time. The results indicated that repeated administration slowed the rate of disappearance of this compound.

It seems reasonable to consider that such a change of rate depends upon an effect on the activity of drug-metabolizing enzymes of fenitrothion treatment. A few reports [6–8] have referred to the effect of organophosphates on liver microsomal drug metabolism and this mechanism cannot be eliminated as one of the important effects of organophosphorus compounds.

In the present report we deal with the inhibition of liver microsomal drug-metabolizing enzymes by a low level of organophosphates.

MATERIALS AND METHODS

Chemicals. The pesticides employed are fenitrothion (Sumithion; *O,O*-dimethyl *O*-3-methyl-4-nitrophenyl phosphorothioate), an oxygenated metabolite of fenitrothion (Sumioxon; *O,O*-dimethyl *O*-3-methyl-4-nitrophenyl phosphate), parathion (*O,O*-dimethyl *O*-4-nitrophenyl phosphorothioate), methylparathion (*O,O*-dimethyl *O*-4-nitrophenyl phosphorothioate) and DDVP (*O,O*-dimethyl *O*-2,2-dichlorovinyl phosphate), which were kindly supplied by Sumitomo Kagaku Kogyo Co., Ltd., and diazinon [*O,O*-diethyl *O*-(2-isopropyl-4-methyl pyrimidinyl) (6)-phosphorothioate], which was a gift from Nippon Kayaku Co., Ltd. Glucose 6-phosphate (G-6-P) and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma Chemical Co.

Animals. Male ddY strain mice, weighing 25–28 g, and albino rats of both sexes, weighing 200 g, were used. Phosphorus insecticides were given intraperitoneally at a dose of 100 or 25 mg/kg, dissolved in 0·1 or 0·3 ml of cottonseed oil to mice and rats respectively. As a control, cottonseed oil was injected.

Enzyme preparation. Animals were decapitated and livers were homogenized with 4 vol. of cold 1·15% KCl and fractionated by means of differential centrifugations as described in a previous report [9]. To obtain post-mitochondrial supernatant, the homogenate was centrifuged at 9000 *g* for 30 min. The microsomes and the soluble fraction were prepared by ultracentrifuging the above mentioned 9000 *g* supernatant solution at 105,000 *g* for 60 min using a Hitachi preparative ultracentrifuge.

Assays. For measurement of drug-metabolizing enzyme activity, aminopyrine (5 μmoles) and aniline (5 μmoles) served as substrates. The incubation mixture consisted of 2·0 ml of 9000 *g* supernatant, 1·0 ml of 0·1 M phosphate buffer (pH 7·4), 50 μmoles $MgCl_2$, 50 μmoles nicotinamide, 0·27 μmole NADP,

1·92 μmoles G-6-P and the substrate in a final volume of 5·0 ml. When the influence of insecticides was tested *in vitro*, each insecticide dissolved in 5 μl ethanol was added to the incubation mixture.

Aniline hydroxylation was determined by the method of Kato and Gillette [10] after 30 min of incubation. In the case of aminopyrine demethylation, production of formaldehyde was quantitatively assayed by the method of Cochin and Axelrod [11].

Hexobarbital sleeping time was measured 4 hr after a single administration of fenitrothion. The mice were given 100 mg/kg of hexobarbital sodium, i.p., and the time during which the righting reflex was absent was designated as sleeping time.

The metabolic conversion of parathion was assayed by determination of phosphate triester extracted from the incubation mixture. The incubation mixture consisted of buffer, cofactors and enzyme solution in the same amounts as those used in the assay for aminopyrine demethylation, except that the substrate was parathion (60 μg). After a measured reaction time, the incubation mixture was mixed with 20 ml acetone and centrifuged to remove denatured protein, which was washed again with 20 ml acetone. The acetone layers were concentrated under reduced pressure to yield an aqueous solution, to which was added 20 ml of 2% NaCl solution and 20 ml hexane. Hexane extraction was repeated twice and the pooled hexane layers were dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in 1 ml acetone and analyzed by gas chromatography.

A Shimadzu GC-5A gas chromatograph equipped with a flame thermionic detector was operated at 190° using a 1·5 m glass column containing 5% SE-30 coated on Shimalite W (60–80 mesh). The retention times were 3·74 and 3·1 min for parathion and paraoxon respectively.

RESULTS

Inhibition of drug metabolism in vivo. Figure 1 shows the time-dependent changes of the activity of oxidative metabolism in the mouse liver 9000 *g* supernatant after a single intraperitoneal injection of fenitrothion in a dose of 100 mg/kg. The time course of metabolism of both substrates was similar and maximum inhibition was seen at 4 hr after dosing. The inhibitory actions of a number of organophosphates at 4 hr after administration of each insecticide are compared in Table 1. Sumioxon and DDVP, both P=O compounds, did not show inhibitory activity. The ineffectiveness of P=O analogues is also seen at other time intervals after dosing, as shown in Table 2.

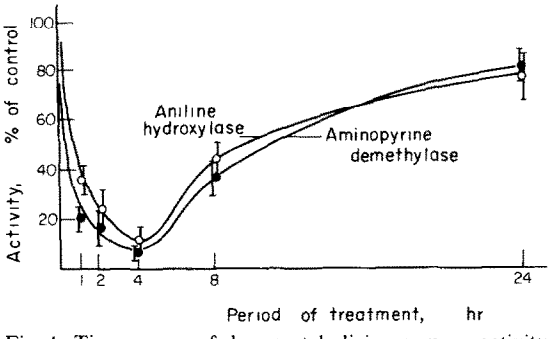

Fig. 1. Time course of drug-metabolizing enzyme activity in mouse liver after injection of fenitrothion. Control and fenitrothion-treated (100 mg/kg i.p.) mice were sacrificed in parallel at each time period. Values are expressed as per cent of control activity at each time period. The vertical bars are standard errors of the mean of three experiments.

Table 1. Effect of organophosphate insecticides on hepatic drug metabolism in mice*

Organophosphate	N-demethylation of aminopyrine Activity	(%)	Hydroxylation of aniline Activity	(%)
Control	12·36 ± 0·55	100	4·53 ± 0·70	100
Fenitrothion	4·66 ± 1·24	37·7	2·28 ± 0·67	50·3
Diazinon	4·93 ± 0·69	39·9	2·52 ± 0·54	55·7
Sumioxon	11·64 ± 0·75	94·1	4·84 ± 0·25	107·0
DDVP	10·09 ± 0·12	81·6	4·23 ± 0·86	93·5

* Organophosphates were given intraperitoneally 4 hr prior to sacrifice in a dose of 25 mg/kg. Activities are expressed as the mean ± S. E. of the metabolites produced in nmoles/mg protein/30 min.

Table 2. Activity of aminopyrine *N*-demethylase in mouse liver after injection of organophosphate*

Treatment	Time after injection (hr) 1	4	6
Control	100	100	100
Fenitrothion	68·4 ± 16·1	39·0 ± 10·4	37·3 ± 4·8
Diazinon	91·5 ± 7·7	41·3 ± 5·8	74·6 ± 0·4
Sumioxon	106·4 ± 3·0	86·4 ± 0·7	90·8 ± 2·2

* Drug was given intraperitoneally in a dose of 25 mg/kg. Values are expressed as activity as per cent of control. The average enzyme activity of the control group in this experiment was 11·95 ± 0·12 nmoles formaldehyde formed/mg protein/30 min.

Table 3. Effect of including organophosphate in incubation mixtures on the activity *in vitro* of hepatic aminopyrine *N*-demethylase from mice*

Organophosphate	Concentration of organophosphate 10^{-4} M	10^{-5} M	10^{-6} M
Fenitrothion	24·70 ± 0·31	41·96 ± 2·33	98·06 ± 1·87
Methylparathion	24·70 ± 0·31	34·10 ± 0·65	82·62 ± 3·35
Sumioxon	111·30 ± 2·22	116·66 ± 2·15	113·50 ± 2·01
DDVP	116·95 ± 3·40	111·72 ± 5·72	106·14 ± 4·44

* Activity was expressed as per cent of control (mean ± S.E.). The activity obtained in the control mice was 11·88 ± 0·85 nmoles formaldehyde formed/mg protein 30 min.

Inhibition of drug metabolism in vitro. Aminopyrine demethylase was significantly inhibited by the addition of organophosphates to the incubation medium, as shown in Table 3. However, P=O compounds were not inhibitory and, moreover, they enhanced the reaction rate somewhat, in contrast to thiophosphates. This observation is compatible with the findings of Stevens *et al* [7].

Effect of fenitrothion on microsomal enzyme kinetics. Fenitrothion competitively inhibited aminopyrine demthylase *in vitro* (Fig. 2a). Since fenitrothion itself is known to serve as a substrate for hepatic microsomal enzymes, it is possible that fenitrothion acts as a competitive inhibitor *in vitro*. When mice were treated *in vivo* with fenitrothion (10 and 25 mg/kg) 4 hr prior to sacrifice, inhibition of aminopyrine demethylase appeared to be noncompetitive (Fig. 2b). This suggests that the inhibition *in vivo* may involve mechanisms other than substrate competition. Further studies are now under way.

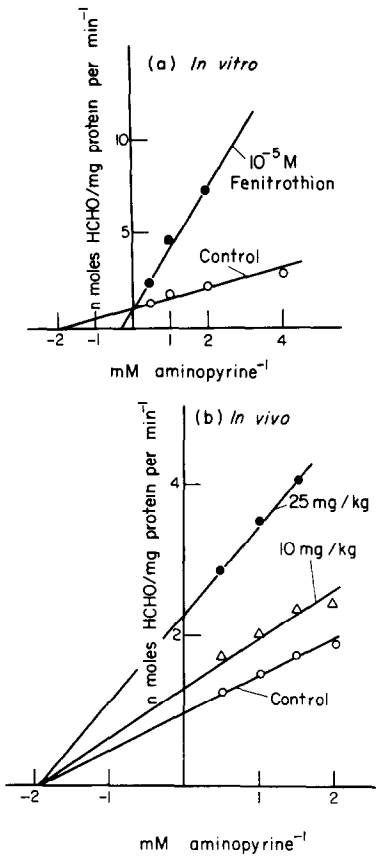

Fig. 2. Lineweaver–Burk plot of the inhibition of aminopyrine *N*-demethylation by fenitrothion in mouse liver.

Species difference: Response of rats. Table 4 shows that treatment of male rats with fenitrothion or diazinon produced only weak inhibition of hepatic drug-metabolizing enzyme activity as compared to the response from treated mice. Female rats, however, were as susceptible as mice. On the other hand, suppression of drug metabolism *in vitro* by thiophosphates was clearly seen in rat liver preparations (Table 5).

Recombination study. It was felt to be possible that a decrease in the concentration of cofactors could be one of the mechanisms responsible for inhibition of drug-metabolizing enzyme activity. To clarify whether limitations in the levels of cofactors supplied to the system brought about the inhibition described above, recombination experiments were carried out with the microsomal and 105,000 *g* supernatant fractions.

As shown in Table 6, the activity of the enzyme dropped markedly when the microsomes were prepared from animals pretreated with fenitrothion. It is obvious, therefore, that the effect of fenitrothion is exerted directly on the microsomes.

Effect of fenitrothion treatment on barbiturate sleeping time. The above results showing the inhibition of drug metabolism led to a test to measure changes of biological half-life of a particular drug using a living animal. The influence of fenitrothion treatment on hexobarbital sleeping time was tested and the results are shown in Table 7.

Four hr after administration of fenitrothion to mice, hexobarbital was injected intraperitoneally. The duration of sleeping was significantly increased by the treatment with fenitrothion. The magnitude of effectiveness is the same as that of SKF-525A, which is well known as a potent inhibitor of drug-metabolizing enzyme activity.

Effect of fenitrothion treatment on metabolic degradation of parathion. From the viewpoint of toxicology, the most important problem is whether the pre-existence of organophosphate *in vivo* influences the metabolic fate of newly administered organophosphates.

Table 4. Effect of organophosphate *in vivo* on hepatic aminopyrine *N*-demethylation in rat liver*

Dose (mg/kg)	Fenitrothion (male rat)	Fenitrothion (female rat)	Diazinon (male rat)
0 (control)	100	100	100
25	106·84 ± 4·38	27·14 ± 1·35	88·81 ± 7·04
50	81·46 ± 20·89	23·94 ± 2·42	85·12 ± 11·85
100	54·58 ± 25·20	21·65 ± 2·52	61·45 ± 8·47

* Organophosphate was injected i.p. 4 hr prior to sacrifice. Values are expressed as remaining activity as per cent of control (means ± S. E.).

Control activities obtained from untreated male and female rats were 11·52 ± 2·58 and 7·68 ± 0·23 nmoles formaldehyde formed/mg protein/30 min respectively.

Table 5. Effect of including organophosphate in incubation mixtures on the activity *in vitro* of hepatic aminopyrine *N*-demethylase from male rats*

Organophosphate	Concentration of organophosphate 10^{-4} M	10^{-5} M	10^{-6} M
Fenitrothion	20·94 ± 3·04	54·63 ± 4·30	85·43 ± 5·82
Methylparathion	18·40 ± 0·63	65·22 ± 3·31	95·84 ± 2·17
Sumioxon	110·42 ± 1·05	101·13 ± 1·05	101·55
DDVP	97·63 ± 1·36	100·73 ± 4·15	89·98 ± 8·05

* Activity was expressed as per cent of control (mean ± S.E.).

The control activity of this experiment was 11·55 ± 0·15 nmoles/mg protein/30 min.

Table 6. Recombination study of subfractions obtained from control and fenitrothion-treated mouse liver*

Substrate	Fraction: Microsomes	Fraction: Supernatant	Activity	Inhibition (%)
Aminopyrine	Control	Control	13·55 ± 0·02	
	Control	Treated	13·70 ± 0·82	
	Treated	Control	5·94 ± 0·10	56
	Treated	Treated	5·29	61
Aniline	Control	Control	7·11 ± 0·59	
	Control	Treated	6·71 ± 0·75	
	Treated	Control	3·35 ± 0·13	53
	Treated	Treated	3·50	51

* Mice were treated with fenitrothion (25 mg/kg) and sacrificed 4 hr after dosing. Activity was expressed as nmoles product formed/mg protein/30 min (mean ± S.E.).

Table 7. Effect of fenitrothion on hexobarbital sleeping time in mice*

Treatment	Sleeping time (min)
Control	50 ± 5
Fenitrothion	215 ± 11

* Four hr after a single administration of fenitrothion (25 mg/kg i.p.), mice were injected intraperitoneally with sodium hexobarbital (100 mg/kg). Values are the times in min ± S. D. during which righting reflex was absent.

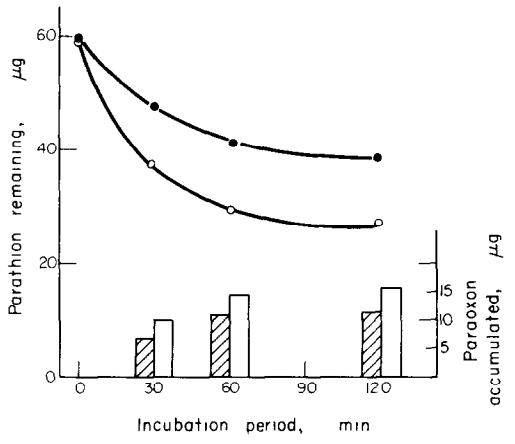

Fig. 3. Oxidative metabolism of parathion by mouse liver preparations. Fenitrothion was intraperitoneally administered to mice in a dose of 25 mg/kg 4 hr prior to sacrifice. Sixty μg parathion dissolved in 10 μl ethanol was added to a reaction mixture consisting of 1·0 ml of 0·1 M phosphate buffer (pH 7·4), 50 μmoles $MgCl_2$, 50 μmoles nicotinamide, 0·27 μmole NADP, 1·92 μmole G-6-P and 2·0 ml of 9000 *g* supernatant fraction prepared from homogenate of control and treated mice liver. The amount of parathion and paraoxon were determined by means of gas chromatography after the conventional extraction as described in text. Each value represents the average of three measurements. Parathion remaining: control, — ·—○—·—; treated, ——●——. Paraoxon accumulated: control, □; treated ▨

Figure 3 shows the rate of metabolism of parathion in liver preparations obtained from control and fenitrothion-treated animals. Pretreatment of mice with 25 mg/kg of fenitrothion suppressed oxidative degradation of parathion, resulting in greater retention in the reaction medium.

DISCUSSION

Among the many actions of organophosphate insecticides on various enzymes and biological components, one of the most important for chronic toxicological evaluation is the effect of the residual levels of organophosphates. The level of several ppm of organophosphates is equivalent to a concentration of *ca.* 10^{-5} M.

This paper demonstrates that the thiophosphate insecticides employed in these experiments inhibit the microsomal oxidative metabolism of aminopyrine and aniline in mice both *in vitro* and *in vivo*.

With fenitrothion inhibition *in vivo* was seen even at a concentration lower than 10^{-5} M. It is reasonable to speculate that the inhibition *in vitro*, which was competitive with substrate, may occur because thiophosphates may function as alternative substrates [7, 8, 12–14].

DDVP and Sumioxon, oxygenated metabolites of fenitrothion, were ineffective as inhibitors of microsomal metabolism *in vitro*. This result is compatible with that of Gandu Rao and Anders [8], who demonstrated that paraoxon and malaoxon added *in vitro* were very poor inhibitors of ethylmorphine metabolism in rat liver microsomes, and suggested that the lack of inhibitory action is attributable to their failure to serve as substrates for the microsomal mixed-function oxidase system.

The inhibitory effect of thiophosphates *in vitro* on drug metabolism is well documented, as mentioned above, but the mode of action of organophosphates on hepatic microsomal drug metabolism in the intact animal is not yet clarified sufficiently. Only alterations of hexobarbital sleeping time [6–8] and zoxazolamine paralysis time [8] have been tested as parameters of response *in vivo*.

In this report the time-dependent changes of the activity of hepatic drug-metabolizing enzyme were investigated and maximum inhibition was demonstrated at 4 hr after injection, irrespective of the type of chemical structure of the thiophosphates examined.

Kinetic studies using the liver preparation from mice treated with fenitrothion *in vivo* indicated that the inhibition *in vivo* was noncompetitive. Further, the recombination of microsomal and soluble fractions prepared from normal and treated animals indicated that the inhibitory effect *in vivo* did not appear in the soluble fraction but only in microsomes. While the detailed mechanism of inhibition obtained by treatment *in vivo* is still obscure, the observations mentioned above indicate that inhibition *in vivo* is not attributable to substrate competition caused by the free organophosphates existing in cytoplasm, but rather to some modification of microsomal membrane or microsomal components. The possibility of microsomal modification through phosphorylation can be excluded by the lack of effectiveness of oxygenated compounds *in vivo*.

Species difference was not shown *in vitro* but was clearly demonstrated in the experiment with organophosphates given *in vivo*. The failure to obtain prolongation of hexobarbital sleeping time by administration of malathion to the rat *in vivo* [8] is quite likely from this result. The weak response of rats may be due at least partly to the more rapid metabolic transformation of thiophosphates in rat liver than in mouse liver.

The alteration of biological half-life of thiophosphate insecticides by pretreatment with thiophosphate itself is quite probable, since the oxidative degradation of parathion in liver preparation was slowed by pretreatment with fenitrothion. The amount of paraoxin in the reaction medium apparently decreased as a result of fenitrothion treatment. Though the amount of paraoxon may be controlled by both oxidative conversion of P=S to P=O and hydrolytic degradation of paraoxon, the decreased accumulation of paraoxon could be a reflection of inactivated oxidative metabolism.

Murphy [15] reported that repeated administration of malathion causes suppression of malathion metabolism because of carboxyesterase inhibition by malathion itself. Therefore, in the same manner, the repeated administration of thiophosphate may produce the slowing of metabolic degradation of thiophosphates. The results obtained from our preliminary experiment presented at the beginning of this paper can be explained by this concept. The detailed investigation, including the half-life in the living animal, effect of the route and frequency of dosing, and the comparison of various metabolic pathways proposed for each thiophosphate, will be reported elsewhere.

REFERENCES

1. A. G. Karczmar, *International Encyclopedia of Pharmacology and Therapeutics*, Section 13, Vol. 1, p. 20. Pergamon Press, Oxford (1970).
2. A. C. Frazier, in *Annual Review of Pharmacology* (Ed. H. W. Elliott), Vol. 7, p. 332. Annual Reviews, Palo Alto (1967).
3. A. Adrian, *Selective Toxicity*, p. 280. Wiley, New York (1965).
4. Report of the Secretary's Commission on Pesticides and Their Relationship to Environmental Health. U.S. Dept. of Health, Education and Welfare (1969).
5. M. Uchiyama, *Third Int. Symp. on Chemical and Toxicological Aspects of Environmental Quality*, Tokyo, p. 25 (Nov. 1973).
6. J. T. Stevens, R. E. Stitzel and J. J. McPhillips, *Life Sci.* **11**, part II, 423 (1972).
7. J. T. Stevens, R. E. Stitzel and J. J. McPhillips, *J. Pharmac. exp. Ther.* **181**, 576 (1972).
8. H. R. Gandu Rao and M. W. Anders, *Bull. environ. Contam. Toxic.* **9**, 4 (1973).
9. Y. Endo and M. Uchiyama, *Biochem. Pharmac.* **19**, 2839 (1970).
10. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150**, 285 (1965).
11. J. Cochin and J. Axelrod, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
12. A. Rubin, T. R. Tephly and G. J. Mannering, *Biochem. Pharmac.* **13**, 1007 (1967).
13. S. D. Murphy and K. P. Dubois, *Fedn Proc.* **16**, 324 (1957).
14. R. H. Welch, W. Levin and A. H. Conney, *J. Pharmac. exp. Ther.* **155**, 167 (1967).
15. S. D. Murphy, *J. Pharmac. exp. Ther.* **156**, 352 (1967).