Studies on the Selective Toxicities of Organic Phosphorous Insecticides (III), The charactors of enzyme system in cleavage of methyl parathion to desmethyl parathion in the supernatant of several species of homogenates (Part I). Jun-ichi Fukami* and Takashi Shishido (The 1st laboratory of Pesticides, The Institute of Physical and Chemical Research, Komagome, Tokyo, and Division of Agricultural Chemicals, National Institute of Agricultural Sciences, Nishigahara, Tokyo) (Received July 26, 1963. *Botyu-Kagaku*, 28, 77 1963.

12. 有機リン殺虫剤の選択的殺虫作用（第3報） 各種生物体の上清画分に存在する有機リン殺虫剤の脱メチル化酵素について（その 1）

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前報（宍戸・深見 1963）では脱メチル化反応がアルキルアリルチオフォスフェート（或はアルキルアリルフォスフェート）殺虫剤の選択性に関係していることが推量された．本論文では，p^{32} 標識メチルパラチオンおよびパラオクソンを使用して種々の条件について各種生物体の細胞分画における上清画分を材料としてこの反応を検討した．反応成立のための協力物質，至適 pH，阻害剤，イオンの影響，嫌気的条件等について観察した．その結果この反応はあきらかに酵素反応であり，一部 SH 系酵素が介在していることがわかつた．またこの反応は高等動物の肝臓の細胞分画における上清画分に最も強く，こん虫全体のホモジェネートの細胞分画における上清画分，体液或は体液の各細胞画分ではきわめて弱いことがわかつた．またこの論文では，既知のパラオクソンを分解する A-エステラーゼおよび，パラオキソナーゼとこの反応との異同について論じた．

It has been shown that an enzyme present in the rabbit serum hydrolyzes diisopropyl fluorophosphate(DFP)to diisopropyl phosphoric acid and hydrofluoric acid[8] and diethyl *p*-nitrophenyl phosphate (paraoxon) to diethyl phosphoric acid and *p*-nitrophenol[1]. Aldridge[2,3] concluded that the paraoxon-hydrolyzing enzyme in serum was identical with A-esterase which was known to hydrolyze *p*-nitrophenyl acetate but not to be inhibited by paraoxon. Later, several investigators[9,10] have confirmed the observation of Aldridge Main[6,7] purified an enzyme that hydrolyzed paraoxon in the sheep serum, and showed that the paraoxon-hydrolyzing enzyme was different from A-esterase. Thus, he called the enzyme paraoxonase to distinguish from the other A-type esterase. Recently Hodgson & Casida[5] examined hydrolysis of p^{32}-DDVP by the mitochondria and soluble enzyme. In the mitochondria the only significant hydrolysis occurred at the *P-O*-vinyl bond, whereas in the soluble fraction both the *P-O*-vinyl and *P-O*-methyl bonds were attacked.

In the previous report, Shishido & Fukami[13] have demonstrated that the reaction system to hydrolyze methyl parathion, sumithion and methyl paraoxon to desmethyl parathion, desmethyl sumithion and desmethyl paraoxon was located in the supernatant of rat liver homogenate. It was not yet known whether the reaction to degrade methyl parathion to desmethyl parathion in the supernatant of rat liver homogenate is enzymatic or not.

The present experiment was undertaken to study systematically the reaction system from methyl parathion to desmethyl parathion in the tissues of rat, guinea pig, rabbit and several insects.

Materials and Methods

Male and female rats(200 to 250 g), guinea pigs (500 to 700 g)and rabbits (1. 5 to 2. 5 kg)were used. The insects used were larvae of the rice stem borer, *Chilo suppressalis*, the adults of the American cockroach, *Periplaneta americana*, both of which were reared in the same way as described previously[13], and the larvae of the horn beetle, *Xylotrupes dichotomus*, which were collected in the field.

P^{32}-labeled methyl parathion was provided by Sumitomo Chemical Co., Ltd. P^{32}-labeled methyl paraoxon was synthesized by the method of Saka-

* This is an account of investigations performed during his stay at Division of Entomology, National Institute of Agricultural Sciences.

moto, *et al*[12]. They were purified and emulsified as described before[4]. NADP, NADPH, NAD, NADH, and GSH were obtained from Sigma Chemical Co., Ltd.

Preparation of subcellular fractionation: The homogenates of the tissues were fractionated as described previously[4,13]. The supernatants were mainly used for experiments. Incubation of homogenate, identification of metabolites, and determination of total nitrogen were made as described previously[4,13].

It is reasonable to assume that the values of radioactivity of water extractable metabolites are indicatives of the amount of desmethyl compounds produced by the degradations of methyl parathion, methyl paraoxon and sumithion[13]. For this reason, the radioactivities of water extractable metabolites were counted to estimate degradation.

Results and discussion

Time course of reaction: Figure 1 shows the time course of degradation of methyl parathion as the values of radioactivity in water extractable metabolites by the supernatant of rat liver homogenate.

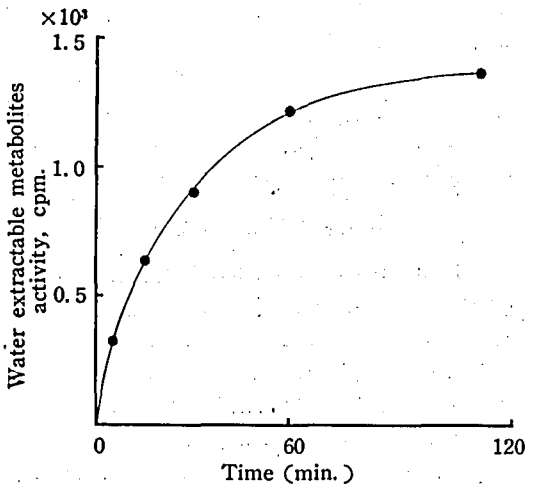

Fig. 1 Rate of degradation of methyl parathion by supernatant of rat liver homogenate (as water extractable metabolites). 360 μg insecticides.

* The following abbreviations are used in this paper: NADP, nicotineamide-adenine dinucleotide phosphate; NADPH, reduced form of NADP; NAD, nicotineamide-adenine dinucleotide; NADH, reduced form of NAD; GSH, reduced glutathion.

Effect of cofactors: The degradation of methyl parathion in the supernatant of rat liver homogenate was little affected by the addition of NADPH, NADP, NADH, NAD or nicotineamide (Table 1).

Optimum pH: The effects of varying the hydrogen ion concentration on the degradation of methyl parathion in the supernatant of rat liver homogenate were studied using citric-HCl buffer,

Table 1. Effects of several cofactors on water extractable metabolites of methyl parathion by the supernatant of rat liver homogenate. 360 μg insecticides.

Addition	water extractable metabolites μg/120min./mg N (Calculated as methyl parathion)
Sup	30.8
Sup+Ni	31.2
Sup+Ni+NAD	32.4
Sup+Ni+NAD+NADH	33.2
Sup+Ni+NADP	32.0
Sup+Ni+NADP+NADPH	33.2

Sup: Supernatant
Ni: 7.2×10^{-3} M Nicotineamide
NAD: 1.5×10^{-4}M NAD
NADH: 1.5×10^{-4}M NADH
NADP: 1.5×10^{-4}M NADP
NADPH: 1.5×10^{-4}M NADPH
(final concentration)

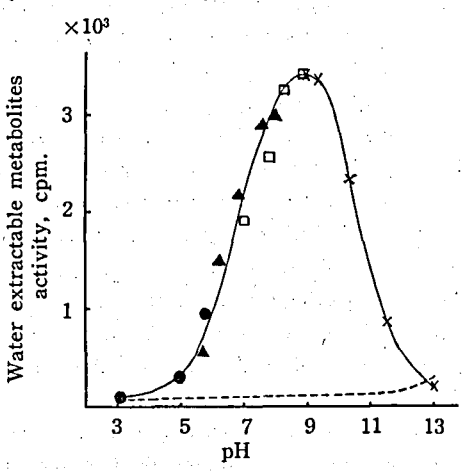

Fig. 2 The activities of water extractable metabolites of methyl parathion by supernatant of rat liver homogenate at different pH values. 360 μg insecticides.
—●—●— citric-HCl (or NaOH) buffer
—▲—▲— phosphate buffer
—□—□— Tris buffer
—×—×— glycine-NaOH buffer
..... No enzymatic activity

Table 2. Effects of several inhibitors on water extractable metabolites of methyl parathion by supernatant of rat liver homogenate. 360 μg insecticides.

Inhibitor added	Final concentration (M)	Percentage inhibition on the production water extractable metabolite
Antimycin A	10^{-5}	0
Rotenone	5×10^{-5}	0
Potassium cyanide	10^{-2}	0
Sodium Fluoride	10^{-1}	12
Sodium monofluoroacetate	10^{-2}	0
Phenyl Mercuric acetate (PMA)	10^{-3}	97.4
(10^{-3}M PMA+6×10^{-2}M GSH)		91.0
PMA	10^{-4}	49.6
(10^{-4}M PMA+6×10^{-2}M GSH)		0
p-chloromercuric benzoate (PCMB)	10^{-2}	95.3
PCMB	10^{-3}	53.3
(10^{-3}M PCMB+6×10^{-2}M GSH)		2.5

citric-NaOH buffer, phosphate buffer, Tris buffer and glycine-NaOH buffer. It is evident from Fig. 2 that the optimum pH lies between 8.5 and 9.5.

Effects of various inhibitors and ions. The results are given in Table 2 and 3. The percentage of inhibition was obtained after a 120 minutes incubation period. The most effective inhibitors were phenyl mercuric acetate and *p*-chloro mercuric benzoate. The addition of Antimycin A, rotenone, potassium cyanide, sodium fluoride or sodium monofluoroacetate had no effect on the activity. The addition of reduced glutathion as an activator to homogenate which was inhibited by phenyl mercuric acetate or *p*-chloro mercuric benzoate restored the activity. This suggest that SH groups are an essential part of this reaction system. The activity was not affected by Ca^{++}, Mg^{++}, Fe^{++}, Fe^{+++}, Mn^{++}, Co^{++}, Ni^{++}, Al^{+++}, Zn^{++}, Ba^{++} and EDTA. However, the activity was inhibited by Cu^{++}.

Table 3. Effects of cations on water extractable tmetabolites of methyl parathion by supernatan of rat liver homogenate. 360 μg insecticides.

Cations added	Final concentration (M)	Percentage inhibition of the production of water extractable metabolite
Ca^{++}	10^{-4}	0.2
Mg^{++}	〃	2.8
Fe^{++}	〃	10.2
Fe^{+++}	〃	9.2
Mn^{++}	〃	10.6
Cu^{++}	〃	50.4
Cu^{++}	10^{-3}	91.3
Co^{++}	10^{-4}	5.5
Ni^{++}	〃	3.7
Al^{+++}	〃	11.3
Zn^{++}	〃	6.8
Ba^{++}	〃	-7.2
EDTA	〃	-2.2

Anaerobic condition: The experiments so far described were carried out in the presence of air. In order to determine whether or not the reaction from methyl parathion to desmethyl parathion in the supernatant of rat liver is an oxidation, incubation was made in the presence of 95% N_2 and 5% CO_2. The water extractable metabolites were identified by ion exchange column chromatography (Fig. 3, Table 4). Most of degradation products in water extractable metabolites were desmethyl parathion (80%) as in the presence of air. This result leads us to the assumption that the reaction from methyl parathion to desmethyl parathion in the supernatant of rat liver is not due to oxidation but due to hydrolysis.

Degradation in various tissues: The values of radioactivity of water extractable metabolites of methyl parathion in the supernatant of homogenate of several tissues of rat are shown in Fig. 4. The liver showed the highest activity which was even greater than the total of the remaining organs studied. The results on rabbit and guinea pig showed the same tendency as that of rat.

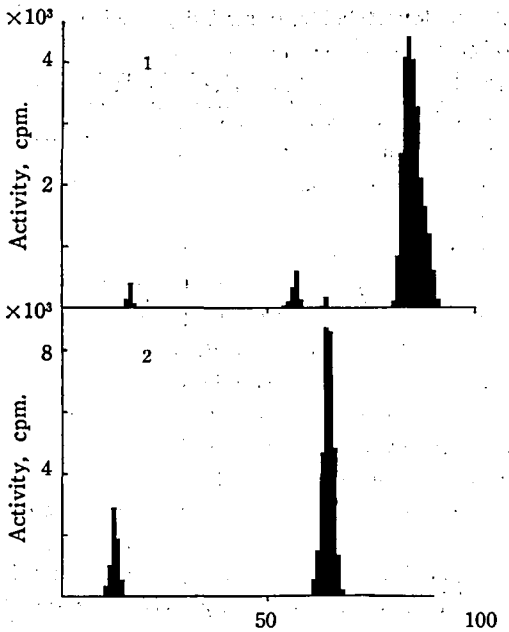

Fig. 3 Degradation products from methyl parathion and methyl paraoxon following incubation with supernatant of rat liver homogenate under anaerobic condition.
(1) Methyl parathion
(2) Methyl paraoxon

Table 4. The activities of the water extractable metabolites of methyl parathion and methyl paraoxon by the subcellular fraction of rat liver homogenate under anaerobic condition.

Insecticides	Water extractable metabolites μg/120 min./mg N (calculated as methyl parathion and methyl paraoxon)
Methyl parathion	
Mitochondria	3.7
Microsome	2.5
Supernatant	30.0
Methyl paraoxon	
Mitochondria	26.7
Microsome	43.4
Supernatant	183.3

Each 3 ml of incubation mixture contained 2.5 ml of homogenate and the indicated concentration: 360 μg insecticides, 2.3×10^{-2}M $NaHCO_3$. incubation: 120 min. at 37°C. 95N_2: 5 CO_2 pH 7.4

Degradation in several species of insect: With the supernatant of the homogenates of rice stem borer larva and cockroach, no principal metabolite of methyl parathion was found[13]; Desmethyl parathion occupied, 23.6% of the degradation product, desmethyl paraoxon 20% and desmethyl sumithion 15%. In addition, the total amount of water extractable metabolites was less than that of mammal. Saito[11] found in his preliminary experiment that in the blood of rice stem borer larvae ethyl parathion was more degraded than in any other orgens. In the present study, water extractable metabolites were examined by incubating P^{32}-labeled methyl parathion with the blood of rice stem borer larvae, or with the subcellular fraction of the blood of horn beetle larvae. But, no activity was found at all.

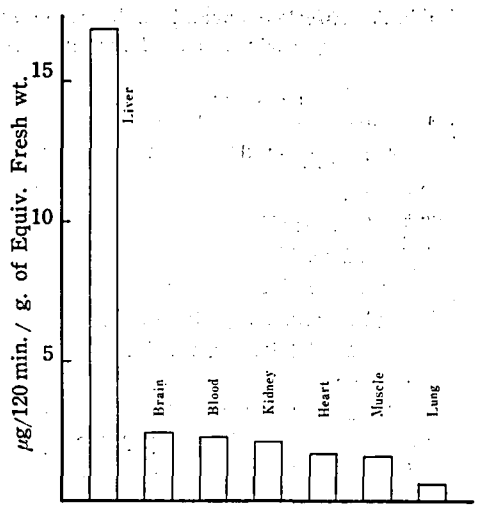

Fig. 4 The activities of water extractable metabolites by the supernatant of several tissue homogenates of rat. 360 μg insecticides.

Based on the foregoing results, it is reasonable to assume that the reaction from methyl parathion to desmethyl parathion in the supernatant of rat liver homogenate is due to hydrolytic enzyme system. It has been known that paraoxon was destroyed by A-esterase and paraoxonase of rabbit serum, and was hydrolyzed to diethyl phosphoric acid and *p*-nitrophenol.[1,2,3,6,7,9,16] In order to see whether the enzyme system is the same as A-esterase or paraoxonase, an experiment was made by incubating the rabbit serum with P^{32}-labeled methyl parathion and methyl paraoxon. The incubation mixture of this experiment was the same as described by Aldridge[2]. As shown in

Table 5, the value of radioactivity of water extractable metabolites of methyl paraoxon was very large, whereas it was negligible in methyl parathion. It can therefore be assumed that the reaction system from methyl parathion to desmethyl parathion in the supernatant of rat liver homogenate is not the same as A-esterase or paraoxonase.

Table 5. The activities of the water extractable metabolites of methyl parathion and methyl paraoxon by the rabbit serum under anaerobic condition.

Substrates	$\mu l CO_2$/60 min.	water extractable metabolites μg/60 min. (calculated as methyl parathion and methyl paraoxon)
methyl parathion	Nil	Nil
methyl paraoxon	120	910

Each 4 ml of incubation mixture contained 0.5 ml of the rabbit serum and the indicated concentration 3.1×10^{-2}M $NaHCO_3$, 1.6×10^{-1}M NaCl, 1000 μg insecticides, gelatine, 0.1% (w/v). Incubation: 60min. at 37° C. 95 N_2: 5 CO_2. pH 7.7

Hodgson & Casida[5] reported that the enzyme system hydrolyzing *P-O*-methyl bond in DDVP metabolism in the soluble fraction of rat liver homogenate precipitated predominately between 60% and 80% on ammonium sulfate fraction. It is very important to study whether the enzyme system hydrolyzing *P* (*S*)-*O*-methyl or *O* (*O*)-*O*-methyl bond in methyl parathion, methyl paraoxon and sumithion.

Summary

The present experiments were made to study the nature of the reaction system from methyl parathion to desmethyl parathion in the tissues of mammal and insects.

1) The time course of reaction, optimum pH, and the effects of cofactors, various inhibitors and ions, and the lack of oxygen on the reaction system were examined. It is assumed that this reaction in the supernatant of rat liver homogenate is not due to oxidation but due to hydrolysis. It has been concluded that this reaction is a kind of enzyme reaction, SH groups being an essential part.

2) The liver showed the highest degradation activity among several organs of rat studied. The results on rabbit and guinea pig showed the same tendency as that of rat. There was no degradation activity in the blood of rice stem borer larvae, *Chilo suppressalis* and of horn bettle larvae, *Xylotrupes dichotomus*, and in the subcellular fraction of the blood of horn beetle larvae.

3) It appears that the reaction system from methyl parathion to desmethyl parathion in the supernatant of rat liver homogenate is not the same as A-esterase or paraoxonase.

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