were applied to the paper treated with mineral oil. After ascending chromatography the papers were sprayed with 5 per cent aqueous sodium hydroxide and heating at 105°C for ten minute for colour development. Under this condition the phosphate esters and *p*-nitrophenol produced deep yellow which was indicative of *p*-nitrophenate ion. The chloroform extracts of the control flasks produced only a single spot, Rf=0.02. However, the chloroform extract of the flasks containing the microsome of homogenate produced three spots, Rf=0.02(parathion), Rf=0.48 and Rf=0.67. The Rf values for two upper spots were identical with the control spots of paraoxon (Rf=0.48) and *p*-nitrophenol (Rf=0.67) providing additional evidence of enzymatic oxidation of parathion to paraoxon. (Fig. 2.)

Summary

Activation of ethyl parathion (*O*, *O*-diethyl *O*-*p*-nitrophenyl thiophosphate) by slices and tissue homogenates of several organs of the rat and larva of the rice stem borer, *Chilo suppressalis* has been investigated.

1. Ethyl parathion was oxidized *in vitro* by the slices of the kidney and liver to a powerful inhibitor of cholinesterase.
2. The activating system of the rat liver homogenate was located in the washed microsome and supernatant fraction. NAD was necessary for this conversion.
3. The activation system in the whole body, homogenate of the borer larvae existed in the washed microsome as in the rat liver. However, it was not NAD but NADP that was effective as cofactor of this conversion.
4. By silica column and reversed phase paper chromatography, the active anticholinesterase substance in the microsome was identified as paraoxon.

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Studies on the Selective Toxicities of Organic Phosphorous Insecticides (II), The degradation of ethyl parathion, methyl parathion, methyl paraoxon and sumithion in mammal, insect and plant. Takashi SHISHIDO and Jun-ichi FUKAMI* (Division of Agricultural Chemicals, National Institute of Agricultural Sciences, Nishigahara, Tokyo and The 1st Laboratory of Pesticides, The Institute of Physical and Chemical Research, Komagome, Tokyo) Received July 26, 1963. *Botyu-Kagaku*, 28, 69, 1963.

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11. 有機リン殺虫剤の選択的殺虫作用（第2報）. 高等動物，こん虫および植物体における有機リン殺虫剤の *in vitro* における分解について

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高等動物，こん虫および植物体における有機リン殺虫剤の選択性を *in vitro* で明らかにするために，p^{32} 標識エチル，メチルパラチオン，メチルパラオクソン，およびスミチオンを使用して，ラッテ，ニカメイチュウ，ゴキブリ，およびカリフラワーの細胞分画におけるこれら殺虫剤の分解について検討した．

イオン交換クロマトグラフィー等により分解生成物を検索した結果，リン酸，ジアルキルチオリン酸，脱アルキル化合物等が認められた．ミトコンドリア，マイクロゾームの画分では，ラッテ，こん虫共に分解物の種類および生成物の比率に差がほとんど認められなかつた．メチル系殺虫剤のラッテ肝臓上消画分における分解は他の画分に比し，非常に強く，そのほとんどが脱メチル化物であつた．一方こん虫では脱メチル化物はわづかしか認められなかつた．また脱エチル化反応は，ラッテ，こん虫共におきにくい．カリフラワーでは，その細胞の各分画において，これら殺虫剤の分解は殆んど認められなかつた．

In Japan, dialkylaryl phosphorothioate insecticides such as ethyl parathion (*O,O*-diethyl *O-p*-nitrophenyl thiophosphate), methyl parathion (*O,O*-dimethyl *O-p*-nitrophenyl thiophosphate) and sumithion (*O,O*-dimethyl 3-methyl-4-nitrophenyl thiophosphate) are widely used to control the larva of the rice stem borer, *Chilo suppressalis*. Sumithion shows very low toxicity against mammals[9], although its chemical structure is closely similar to those of methyl parathion and ethyl parathion which are highly toxic to mammals. Therefore, it is worth-while to study differences of metabolism of these insecticides in mammal, insect and plant.

It has been assumed that hydrolysis of dialkylaryl phosphate insecticides occurs only at the aryl phosphate bond[1,4]. Plapp and Casida[11] demonstrated that alkyl phosphate hydrolysis of certain dialkylaryl phosphorothioate appears to occur when the dosage is too great to be metabolized in rat through hydrolysis of the aryl phosphate bond. These conclusions are based on *in vivo* experiments in which degradation products are isolated. The nature and distribution of the responsible enzyme systems are not known.

In the present report, the metabolism and nature of the products formed by *in vitro* degradation of ethyl parathion, methyl parathion, methyl paraoxon (*O,O*-dimethyl *O-p*-nitrophenyl phosphate) and sumithion in mammal, insect and plant were studied using homogenate preparations from several tissues.

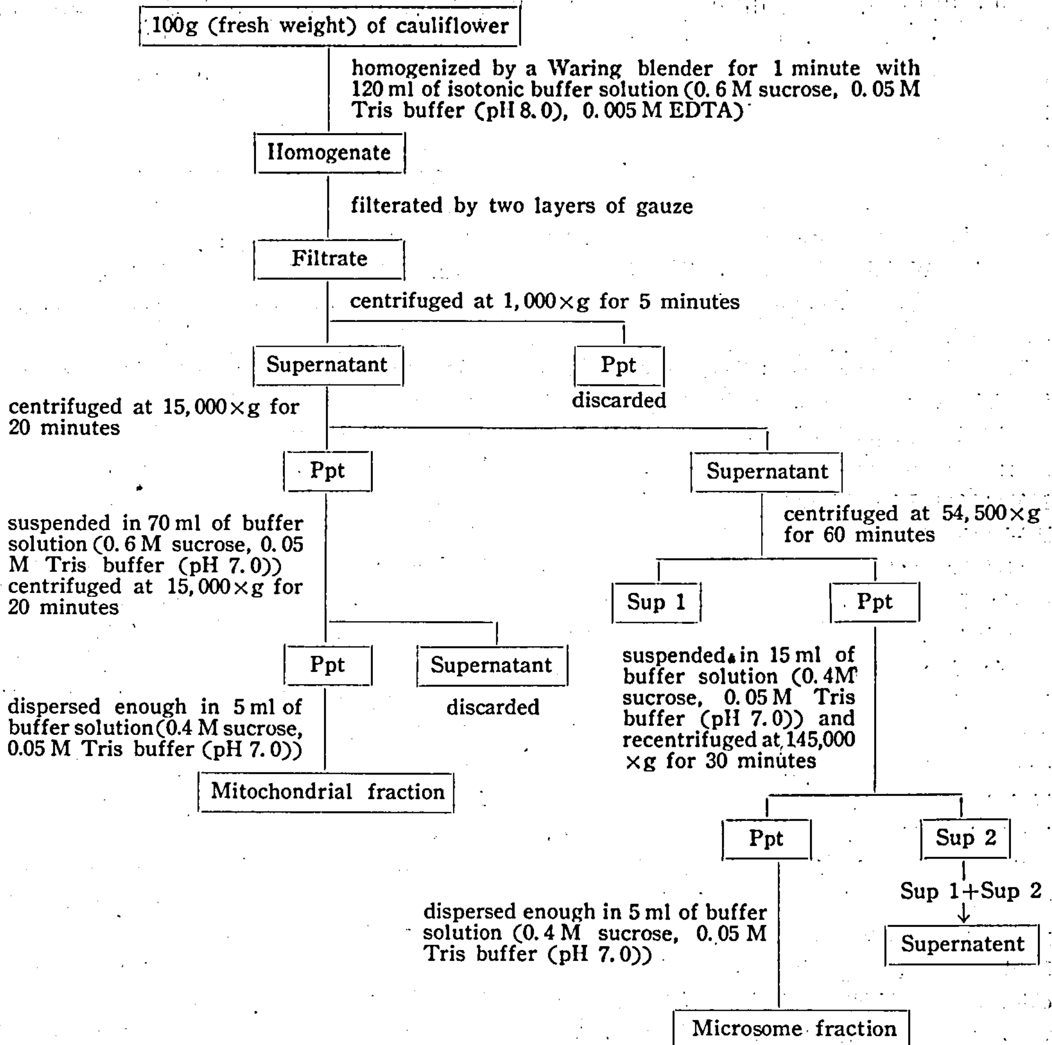
Materials and Methods

Male and female albino rats (200 to 250g), cauliflowers, the larvae of the rice stem borer and the adults of the American cockroach, *Periplaneta americana* were used. The rice stem borer larvae were reared by synthetic diet. The diet was a mixture of the following ingredients:

Ingredient	Amount
Water	40.0ml
Agar	0.6g
Glucose	0.7
Sucrose	0.3
Casein	1.0
Cholesterol	0.02
Cholinechloride	0.2
Brewer's yeast	1.0
McCollum & Simond's Salt	0.2
Rice stem	20.0

The mixture was placed in 200ml Erlenmeyer flasks. The eggs of the borer were collected in Saitama Prefecture. The eggs laid on a piece of paper, were disinfected with 0.1% aqueous solution of mercuric chloride, and placed in each flask at a population of 30 to 40 eggs. Aseptic rearing was carried out in an incubator at 30°C under photoperiod of sixteen hours of light per day. After a rearing period of about thirty days, the larvae were used for experiment. The American cockroaches were fed on baker-yeast at 30°C. The insecticides and their derivatives used in the experiment were prepared according to the description of Plapp & Casida[11] and Kosolapoff[6].

Scheme 1. The flow-sheet of subcellur fractionation in the homogenate of cauliflower



P^{32}-labeled ethyl parathion, methyl parathion and sumithion were provide by Sumitomo Chemical Co., Ltd. These labeled phosphorous insecticides were purified by shaking with chloroform and water containing 1% (w/v) Na_2CO_3, washing, drying and distilling off the chloroform, and then by silica column chromatography. P^{32}-labeled methyl paraoxon was synthesized by the method of Sakamoto, *et al*[12]. Specific activities of P^{32}-labeled ethyl parathion, methyl parathion, methyl paraoxon and sumithion were 5.0mc/g, 17.2mc/g, 18.3mc/g and 4.9mc/g respectively at the time of experiment. Stock solutions were prepared by dissolving the insecticides in absolute alcohol. Emulsifiable concentrates of radioactive insecticides, consisting of 50 parts stock solution and 50 parts emulsifer (TritonX-100) were prepared. The concentrates were diluted by water to give desired concentrations of insecticides. NADP*, NADPH, NAD and NADH were obtained from Sigma Chemical Co., Ltd.

Preparations of subcellular fractionation: The fractionations of homogenates of the rat liver, the

* The following abbreviations are used in this paper: NADP, nicotineamideadenine dinucleotide phosphate; NADPH, reduced form of NADP; NAD, nicotineamideadenine dinucleotide; NADH; reduced form of NAD.

rice stem borer larva and the adult cockroach were carried out by the same methods as described in the author's preceding report[2]. The liver was chosen because it caused highest degradation of phosphorous insecticides among several organs of rat[3]. Because of small sizes of the rice stem borer larva and the adult cockroach, whole insect bodies were used. Subcellular fraction ation of the homogenate of cauliflower was prepared by the method of Wedding & Black[14]. The flow-sheet of preparation of cauliflower is summarized in Scheme 1.

Incubation of homogenate: This was carried out in a Warburg apparatus. The incubation mixture consisted of 2.5 ml of homogenate, 0.1 ml of 2.4×10^{-1}M nicotineamide, 0.1 ml of 2.5×10^{-1}M NAD+NADH (or NADP+NADPH) and 0.3ml of P^{32}-labeled insecticide. The mixture was incubated for two hours at 37°C, with shaking in the presence of air. In mammal, NAD and NADH were used as cofactors. As shown in the previous report[2], the *in vitro* activation of parathion to paraoxon in tissue homogenate of the rice stem borer larvae occurred when NADP was added as cofactor, but not when NAD was used. For this reason, only NADP and NADPH were used as cofactors for the incubation of insect homogenate. In cauliflower, both NADP+NADPH and NAD+NADH were added as cofactors. At the end of incubation period, reaction was stopped by the addition of 1 ml of an aqueous solution containing 10 g trichloroacetic acid in 100 ml of water. The mixture were then macerated in a Waring blender. The macerate were placed for 90 minutes in crushed ice, and 4 ml of chloroform was added. After macerating this mixture in a Waring blender again, chloroform and water extractables were separated by centrifugation, and the radioactivity of each extractable was estimated for unit volume.

Identification of metabolites: The metabolites in the water extractable were identified by means of ion exchange and paper chromatography according to the method by Plapp & Casida[10] and Tomizawa & Sato[13]. The sample solution (pH 4) was placed in the column, and then the top of column was washed with a few ml of distilled water. The column was placed on a fraction collector, and 4 ml eluate per fraction was collected. One ml aliquot of each fraction was transferred to a planchet for radioassay and the content of the planchet was dried up under the mild heat by an infrared lamp. Ion exchange resin was Dowex I-X8 (100-200 mesh, Cl type). The eluting solvents were as follows;

I. pH 2 to pH 1 HCl, 200ml
II. pH 1 HCl plus methanol (1:3) to 1 N HCl plus methanol (1:3), 100ml
III. 1 N HCl plus methanol (1:3) to concentrated HCl, water and methanol (1:1:6), 100ml

Identification of eluted metabolites was based on the comparison with the result of an ion exchange chromatography carried out with the known compounds. In order to secure the identification of the metabolites, the following paper chromatography system was used;

1. Filter paper impregnated with 5% silicone 550 in hexane, mobile solvent, the upper from a mixture of ethyl alcohol 10 parts, chloroform 10 parts and water 6 parts by volume[8].
2. Filter paper without any treatment, mobile solvent, a mixture of isopropyl alcohol 75 parts and concentrated ammonium hydroxide 25 parts[10].

In the case of radioactive metabolites, the paper chromatography was carried out with the concentrates of water soluble and chloroform soluble fractions, and the detection of spots was done by autoradiography.

Determination of total nitrogen: The amount of total nitrogen in the homogenate preparation was determined by micro-Kjeldahl method after digestion by sulfuric acid.

Results and Discussion

Degradation of methyl parathion, methyl paraoxon and sumithion in the subcellular fractions of rat, rice stem borer larva, adult cockroach and cauliflower: The results are shown in Table 1. The order of activity for the degradation of methyl parathion, methyl paraoxon and sumithion by rat liver homogenate was supernatant>mitochondria≒washed microsome. Methyl paraoxon was almost completely degradated in the supernatant. In insect there are little difference in the amounts of degradation products between supernatant, washed microsome and mitochondria.

There are no difference in the water extractable metabolite of methyl parathion between the homogenate of the rice stem borer larva and of adult of the American cockroach. No degradation of methyl parathion and methyl paraoxon occurred in the cauliflower.

Hodgson & Casida[6] reported that in the rat liver homogenate, DDVP was hydrolyzed by the soluble and mitochondrial fractions but not by the microsomes, and desmethyl DDVP was hydrolyzed by the soluble fraction but not by the microsome or mitochondria. This agrees with the present result in that degradation occurs in the soluble fraction of rat liver homogenate. On the other hand, Matumura & Brown[7] reported that in the hydrolysis of malathion and malaoxon in the homogenate of *Culex tarsales* larvae the order of the activities of carboxyesterase and phosphatase was mitochondria >microsome>supernatant. Their result is rather opposite to the present one with the homogenates of rice stem borer larvae and the adult cockroaches.

Degradation of ethyl parathion, sumithion and methyl parathion on the subcellular fraction of rat liver and rice stem borer larva: As the specific activity of P^{32}-labeled ethyl parathion was low, its degradation in the homogenates of rat liver and rice stem borer larva was investigated by incubating with 1 mg per 3 ml system. For comparison, the degradations of sumithion and methyl parathion were examined at the same concentration as that of ethyl parathion. As shown in Table 1, although sumithion and methyl parathion were effectively degradated at the concentration of 1 mg per 3 ml system, the degradation of ethyl parathion was very small. In the case of the rice stem borer larva, the degradation of ethyl parathion was very small in amount.

Separation of water extractable metabolites: The water extractable metabolites were identified by the ion exchange chromatography. As shown in Fig. 1 and Table 2, five peakes of metabolites were found for methyl parathion and sumithion,

Table 1. Amounts of degradation of methyl parathion, methyl paraoxon and sumithion by the subcellular fraction of several tissues.

Insecticide	μg/120 min. /N mg as metabolites on water extractable (calculated as ethyl parathion, parathion, methyl paraoxon or sumithion). (dosage: 360μg)			
	$(CH_3O)_2P(S)-O-C_6H_4-NO_2$	$(CH_3O)_2P(O)-O-C_6H_4-NO_2$	$(CH_3O)_2P(S)-O-C_6H_3(CH_3)-NO_2$	$(C_2H_5O)_2P(S)-O-C_6H_4-NO_2$ *
	Rat			
Mitochondria	2.1	19.8	1.9	0.54
Microsome	3.6	43.1	3.6	1.9
Supernatant	23.4 (90.3)**	119.4	19.9 (107.3)**	2.4
	Rice stem borer			
Mitochondria	1.4		2.1	0.39
Microsome	2.0		2.8	0.96
Supernatant	2.8		4.9	0.31
	Cockroach			
Mitochondria	0.23	0.82		
Microsome	0.38	1.3		
Supernatant	1.0	2.6		
	Cauliflower			
Mitochondria	Nil	Nil		
Microsome	Nil	Nil		
Supernatant	0.1	0.1		

* This dosage is 1 mg, ( )**: 1 mg is used as a dosage.

Table 2. Water extractable metabolites produced by the subcellular fraction to radioactive insecticides: Amounts as percentage of total recovered (dosage: 360μg.)

Insecticide	Rat Mit.	Rat Mic.	Rat Sup.	Rice stem borer Mit.	Rice stem borer Mic.	Rice stem borer Sup.	Cockroach Sup.
A $(CH_3O)_2P(S)\text{-}O\text{-}C_6H_4\text{-}NO_2$							
unknown (peak 1)	2.0	2.0	0.5	2.4	3.7	5.7	
H_3PO_4 or H_3PO_3S (peak 2)	35.6	67.5	9.2	52.1	50.3	38.9	
$(CH_3O)_2P(S)\text{-}OH$ (peak 3)	14.2	16.7	5.3	19.2	13.6	23.6	
$(CH_3O)(OH)P(O)\text{-}O\text{-}C_6H_4\text{-}NO_2$ (peak 4)	8.5	7.8	1.5	5.5	5.3	4.2	
$(CH_3O)(OH)P(S)\text{-}O\text{-}C_6H_4\text{-}NO_2$ (peak 5)	33.3	9.2	81.8	17.3	17.1	23.6	
B $(CH_3O)_2P(O)\text{-}O\text{-}C_6H_4\text{-}NO_2$							
unknown (peak 1)	2.0	2.0	2.0				
H_3PO_4 or H_3PO_3S (peak 2)	78.0	90.0	8.0				80.0
$(CH_3O)(OH)P(O)\text{-}O\text{-}C_6H_4\text{-}NO_2$ (peak 3)	20.0	8.0	90.0				20.0
C $(CH_3O)_2P(S)\text{-}O\text{-}C_6H_3(CH_3)\text{-}NO_3$							
unknown (peak 1)	1.5	1.6	0.7	1.5	2.3	4.3	
H_3PO_4 or H_3PO_3S (peak 2)	35.6	66.5	11.2	69.8	63.4	53.9	
$(CH_3O)_2P(S)\text{-}OH$ (peak 3)	26.1	21.8	5.3	16.1	20.6	20.2	
$(CH_3O)(OH)P(O)\text{-}O\text{-}C_6H_3(CH_3)\text{-}NO_2$ (peak 4)	4.1	4.4	2.3	3.4	3.0	2.8	
$(CH_3O)(OH)P(S)\text{-}O\text{-}C_6H_3(CH_3)\text{-}NO_2$ (peak 5)	39.7	3.7	78.6	6.3	7.4	15.6	
D $(C_2H_5O)_2P(S)\text{-}O\text{-}C_6H_4\text{-}NO_2$*							
unknown (peak 1)							
H_3PO_4 or H_3PO_3S (peak 2)	44.5	73.8	18.7				
$(C_2H_5O)_2P(S)\text{-}OH$ (peak 3)	42.6	20.8	56.3				
$(C_2H_5O)(OH)P(O)\text{-}C_6H_4\text{-}NO_2$ (peak 4)	4.8	1.6	2.0				
$(C_2H_5O)(OH)P(S)\text{-}C_6H_4\text{-}NO_2$ (peak 5)	5.6	0.7	17.1				

* This dosage is 1 mg Mit: Mitochondria, Mic: Microsome, Sup: Supernatant

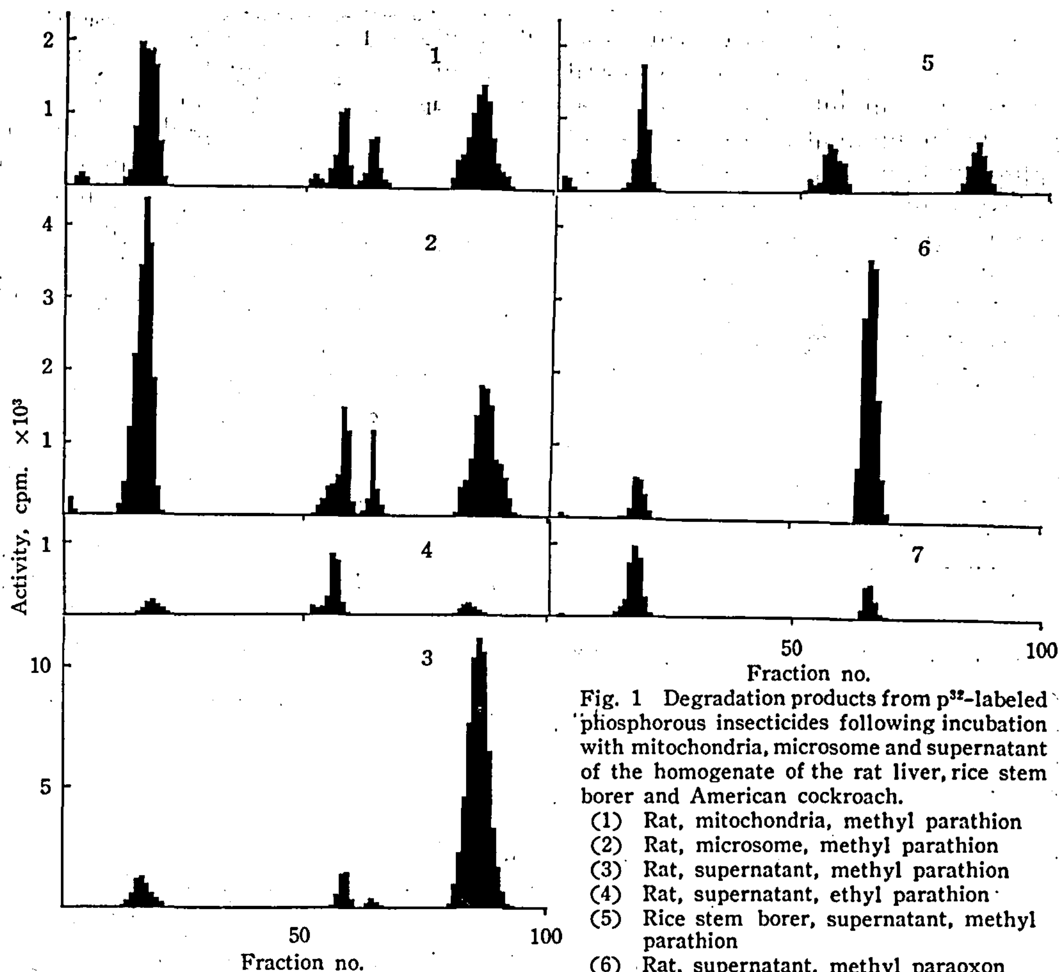


Fig. 1 Degradation products from P^{32}-labeled phosphorous insecticides following incubation with mitochondria, microsome and supernatant of the homogenate of the rat liver, rice stem borer and American cockroach.
(1) Rat, mitochondria, methyl parathion
(2) Rat, microsome, methyl parathion
(3) Rat, supernatant, methyl parathion
(4) Rat, supernatant, ethyl parathion
(5) Rice stem borer, supernatant, methyl parathion
(6) Rat, supernatant, methyl paraoxon
(7) Cockroach, supernatant, methyl paraoxon

four peaks for ethyl parathion and three peakes for methyl paraoxon. With the supernatant of rat liver homogenate, the principal metabolites of methyl parathion, methyl paraoxon and sumithion were *O*-methyl *O*-*p*-nitrophenyl thiophosphoric acid (desmethyl parathion), *O*-methyl *O*-*p*-nitrophenyl phosphoric acid (desmethyl paraoxon) and *O*-methyl 3-methyl 4-nitrophenyl thiophosphoric acid (desmethyl sumithion) respectively (81. 8, 90. 0 and 78. 6%) with remaining radioactivity appearing in thiophosphoric or phosphoric acid, dimethyl thiophosphoric acid, desmethyl paraoxon, desmethyl sumithion oxide. However, with the supernatant from the rice stem borer larva and adult cockroach, these principal metabolites were not found in such large amounts as in the case of the rat liver ; desmethyl parathion was 23. 6%, desmethyl paraoxon was 20%, and desmethyl sumithion was 15.6%. Amounts of their degradation products were considerably different from those in the rat liver. In the experiment of Table 1 and 2, NAD and NADH were used as cofactors for the rat liver homogenate, whereas in insect NADP and NADPH were added. It may be that the difference of degradation products in the supernatant between insect and rat liver is due to the difference in cofactor. This possibility is however excluded by the finding that no change in the amounts of degradation products was found when NADP+NADPH was used in place of NAD+NADH in the rat liver homogenate[3].

The toxicity of ethyl parathion to rat is higher than that of methyl parathion and sumithion. The production of desethyl parathion in the

supernatant was very slight, 17.1% (Table 2). Hodison & Casida[5] examined the nature of initial hydrolysis of DDVP by the mitochondrial and soluble enzyme preparations. The enzyme hydrolyzing the *P-O*-methyl precipitated predominately between 60 and 80% saturation of ammonium sulfate fractionation of the soluble liver fraction, while *P-O*-vinyl hydrolyzing esterase precipitated between 40 and 60% saturation. In the present investigation, it was shown that the reaction systems cleaving *P* (*S*) *O*-methyl and *P* (*O*) *O*-methyl of methyl compounds existed in the supernatant of rat liver homogenate. Therefore, it is of interst to see whether these reactions are due to the same enzyme system as that of DDVP. It is also necessary to show the difference of degrading reactions between metabolism of *P*(*S*)*O*-methyl lindage and that of *P*(*O*)*O*-methyl lindage.

Summary

The *in vitro* degradations of ethyl parathion (*O,O*-diethyl *O-p*-nitrophenyl thiophosphate), methyl parathion (*O,O*-dimethyl *O-p*-nitrophenyl thiophosphate), methyl paraoxon (*O,O*-dimethyl *O-p*-nitrophenyl phosphate) and sumithion (*O,O*-dimethyl 3-methyl-4-nitrophenyl thiophosphate) in the homogenate preparations of mammal, insect and plant have been investigated.

The order of activity to degrade methyl parathion, methyl paraoxon and sumithion, to water extractable metabolites in the rat liver homogenate was: supernatant>mitochondria≒washed microsome. However, in the case of ethyl parathion, the activity of degradation in the supernatant of the rat liver homogenate was very small compared with that of methyl parathion and sumithion. In insect, there was little difference between supernatant, washed microsome and mitochondria in degradation activity. In plant, no degradation of methyl parathion and methyl paraoxon occurred.

These water extractable metabolites were identified by ion exchange chromatography, and five peaks of metabolites were found. Four of them were identified as phosphoric or thiophosphoric, dialkylthiophosphoric, desalkyl thiophosphoric, and desalkyl phosphoric acid, the remaining one being unknown. With the supernatant of the rat liver homogenate, the principal metabolites of methyl parathion, methyl paraoxon and sumithion were desmethyl parathion, desmethyl paraoxon and desmethyl sumithion, respectively. In the case of ethyl parathion, the production of desethyl parathion was very slight. With the supernatant of the homogenate of insects, these metabolites were found not to be principal.

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