manuscript. They are also grateful to Dr. K. Ozaki, National Institute of Agricultural Sciences, to Messrs. H. Uehara and K. Kosaka, Kagawa Agricultural Experiment Station, and to Mr. M. Nakada, Shizuoka Agricultural Experiment Station, for supply of testing insects. The technical assistance of Messrs. A. Shiino, H. Tanaka and T. Kitagaki is acknowledged.

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Studies on the Selective Toxicities of Organic Phosphorous Insecticides. (I). Activation of ethyl parathion in mammal and insect (Part 1). 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**, 63, 1963.

10. 有機リン殺虫剤の選択的殺虫作用 第1報. 高等動物およびこん虫におけるエチルパラチオンの活性化について (その 1). 深見順一 (理化学研究所農薬第一研究室)・宍戸孝 (農林省農業技術研究所農薬科) 38. 7. 26. 受理

生体内でパラチオンを活性化せしめる実験については数多くの報告がなされている. この論文においては, これらの実験の追試を, ラッテおよびニカメイチュウを材料としておこない, 新しい知見を得たので報告する. ラッテの臓器の切片による活性化は肝および腎臓において認められたが, 脳および後脚の筋肉では認められなかつた. つぎに肝臓の細胞分画における活性化の実験では, マイクロゾームにおいて最も強く, 上清がこれにつぎ, ミトコンドリアの画分では認められなかつた. またニカメイチュウについては, 直接細胞分画における活性化の実験をおこない, ラッテと同じくマイクロゾームにおいて認められたが, ミトコンドリアでは認められなかつた. しかしこの際ラッテの場合と相違して活性化の条件における助酵素としてニコチンアマイドアデニンディヌクレオタイドリン酸 (NADP ; 助酵素 II) を必要とした. またこれら活性化物はパラオクソンであることをクロマトグラフィーにより同定することができた.

Ethyl parathion (*O, O*-diethyl *O*-*p*-nitrophenyl thiophosphate), when administered to animals, causes a decrease in the cholinesterase levels of blood and tissues which is accompanied by typical signes of acetylcholine poisoning despite its weak potency to inhibit cholinesterase (ChE) *in vitro*.[1,3]

It has been shown that, in the presence of oxygen, ethyl parathion was oxidized by mammalian liver slices to ethyl paraoxon (*O, O*-diethyl *O*-*p*-nitrophenyl phosphate), a highly active inhibitor of ChE.[5,8] The capacity of the liver preparation to activate parathion was completely lost by homogenization. Davison[2] found that activating capacity of ethyl parathion in rat liver

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

homogenates could be restored by adding magnesium and NAD* along with nicotineamide.

This conversion also took place *in vitro* in the presence of several tissues from the American cockroach under aerobic condition, and the reaction was completely prevented by homogenizing the tissues.[7,8] However, several investigators pointed out that the activation of phosphorous insecticides in the homogenate of insect tissues could be restored by adding cofactors; O'Brien[10] could to some extent restore the capacity of the American cockroach gut homogenate to activate malathion by adding NAD, nicotineamide and magnesium.

Fenwick[4] reported that the homogenate of the locust fat body activated shradan without exogenous cofactors and that a particulate fraction required NADPH as a cofactor for activation. Nakatsugawa and Dahm[9] reported that activation of guthion by tissue homogenate from the American cockroach required oxygen and NADPH or other nicotineamide as cofactors.

It remains to be seen whether or not activation of ethyl parathion occurs in insect homogenate in the presence of cofactors. It is therefore of great interest to investigate this problem under a variety of experimental conditions. The present study is concerned with the activation of ethyl parathion by slices and tissue homogenate of several organs of the rat and the rice stem borer larva.

Materials and Methods

Male and female albino rats (200-250g) were sacrificed, and the slices and homogenate of several organs were used. The homogenate of the diapausing larvae of the rice stem borer, *Chilo suppressalis* collected from Miyazaki Prefecture were used.

Ethyl parathion was supplied from Sumitomo Chemical Co., Ltd. The insecticide was purified by shaking with chloroform and water containing 1% (w/v) Na_2CO_3, drying and distilling off the chloroform, and then by silica column chromatography. Stock solution was prepared by dissolving the purified parathion in absolute ethanol. The concentration of the stock solution was 4×10^{-4}M for parathion. Aqueous dilution of this solution was used for the experiments. NADP, NADPH, NAD and NADH were obtained from Sigma Chemical Co., Ltd.

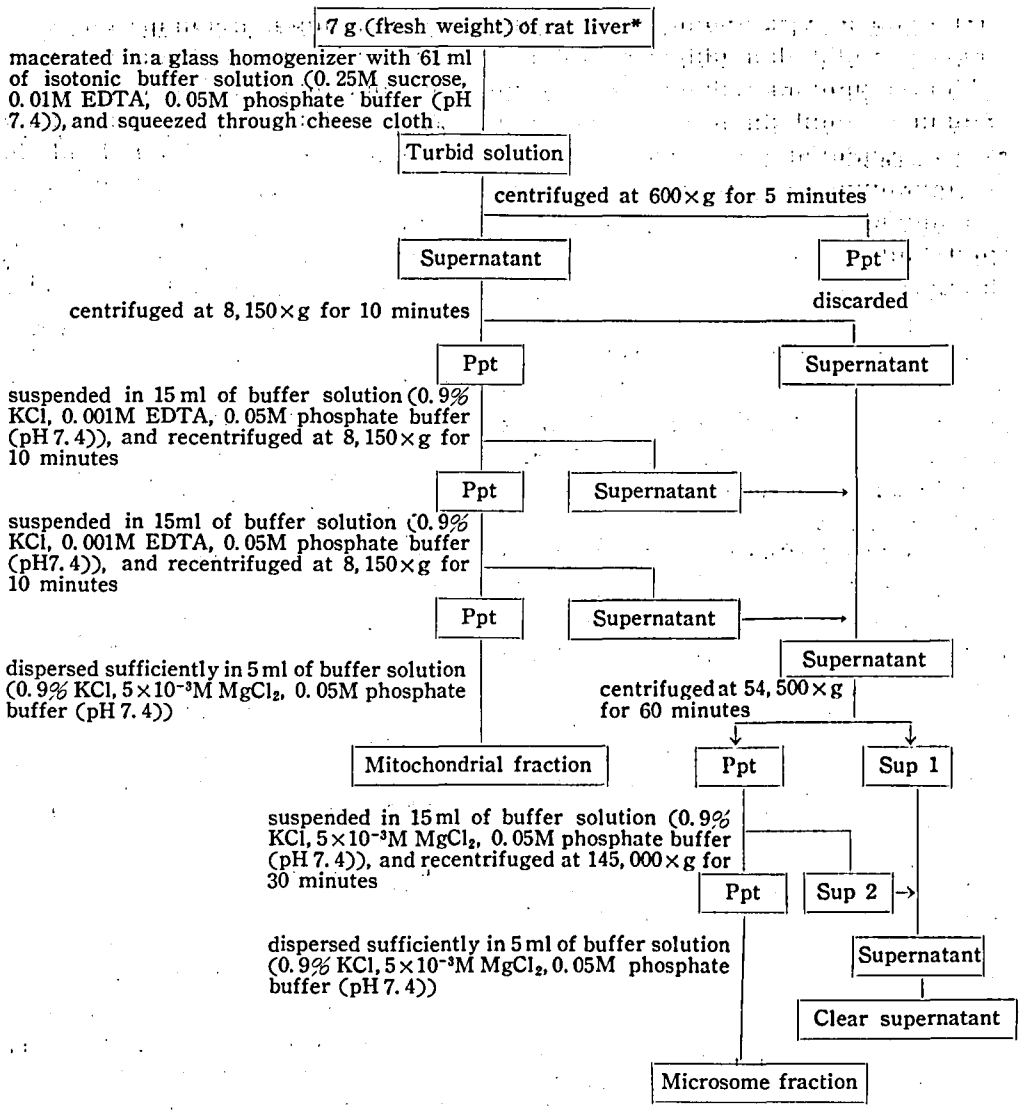
Preparation subcellular fractionation: The tissue homogenates of the rat and the rice stem borer larva were used as cell-free homogenates. The fractionation of the homogenates was carried out according to flow-sheet summarized in Scheme 1. In the rat, liver was mainly used, whereas in insect, the whole body was homogenized. All the procedures were conducted at temperatures not exceeding 4°C.

Activation of parathion by intact slices: The slices (700mg, wet weight) of several organs of rat were incubated in 2.7 ml of Krebs-Ringer phosphate buffer pH 7.4[12] containing 0.1 ml of 0.3M glucose and parathion at a final concentration of 4×10^{-6}M in the presence of oxygen for two hours at 37°C by using the Warburg apparatus. At the end of incubation period, the slices were homogenized in the medium and the suspension was centrifuged at 600×g for 10 minutes. The supernatant was used for anticholinesterase assay. Control was run by adding parathion from the side arm of vessel at the end of incubation period and by blocking the reaction by adding hot water to the preparation.

Activation of parathion by homogenate: The rat liver and insect homogenates were incubated for two hours in the presence of oxygen or air by using the Warburg apparatus. The incubation mixture consisted of 2.5ml of cell-free homogenate, 0.1 ml of nicotineamide, 0.1ml of 5×10^{-3}M nicotineamide-adenine nucleotide, and 0.3 ml of parathion solution. At the end of incubation period, the mixture was rapidly cooled by immersing in ice water, and sample (0.3 ml) was taken for anticholinesterase assay. The experiment for control was the same as those for the slice experiment.

Paper and column chromatographies of parathion activation in the homogenate of rat and rice stem borer larva: Demonstration of active anticholinesterase substance in the homogenate was carried

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



* The procedure for the preparation of subcellular fraction of insect homogenate was almost the same as that of the rat liver except that 22g of the fresh weight of the rice stem borer was homogenized in about four times body weight of the same solution.

Scheme I. The flow-sheet of subcellular fractionation of the homogenate of rat liver.

out by silicic acid column and reversed paper chromatography.

Silicic acid column chromatography: 3 g of silic acid was mixed with 3 ml of 90% methanol (methanol-water 9 : 1) and 15 ml of mobile solvent (*n*-hexane-benzol (85 : 15) mixture saturated with 90% methanol) in a beaker. The mixture was poured into a 1×30 cm chromatographic column. Pressure of 0.5 to 1 lb/in² was applied for a few minutes. The pressure was released, the effluent collected was used to rinse the beaker and the side of the column. Pressure of 0.5 to 1 lb was applied again until the surface of the mobile solvent reached the top of the silicic acid layer. The sample to be chromatographed was dissolved in a small volume of the mobile solvent. One ml of the sample solution was placed on the column without disturbing the surface of silicic acid. The pressure was applied until the level of the sample solution reached the top of silicic

acid. The wall of the column was rinsed with a few ml of mobile solvent, and the pressure was applied again and released when the surface of the mobile solvent reached the top of column. 30 ml mobile solvent was then added under pressure. 2 ml of effluent per fraction was collected.

Reversed phase paper chromatography: Toyo No. 50 filter paper was dipped in a 40% of mineral oil in *n*-hexane. The paper was blotted between two sheets of absorbent paper to remove excess solution, and placed in the air for 1 hour. After the sample solution was applied, chromatography was carried out using the following solvent, the upper phase of a mixture of 1 part chloroform, 2 parts acetone, 2 parts ethyl alcohol and 4 parts water.

Esterase determination: The anticholinesterase assay was carried out manometrically at 37°C in an atmosphere of 5% carbon dioxide and 95% nitrogen. Reading over a period of 30 minutes were used to calculate per cent inhibition of ChE. The contents of the Warburg flask were as follows; 0.3 ml of the sample to be assayed, 1 ml of ChE preparation, 0.7 ml Ringer solution in the main compartment, and 0.5 ml of 0.06M acetylecholine bromide in the side arm. The ChE preparation was prepared by homogenizing the heads of the house flies, *Musca domestica*, in the above-mentioned Ringer solution in the proportion of 10 mg per ml, filtering the homogenate through a cotton plug and centrifuging approximately 600×g for 10 minutes. The supernatant fluid was used as the ChE sourse. The compositions of Ringer solution were 0.15M NaCl and 0.05M $MgCl_2$.

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

Results and Discussion

Production of anticholinesterase substance from ethyl parathion by slice incubation: The slices of several organs of rat were immersed in the presence of oxygen in a Ringer solution to which ethyl alcohol solution of purified ethyl parathion was added to give a final concentration of 4×10^{-6}M. The results are given in Table 1. With the unincubated parathion no appreciable inhibition of ChE was obtained. However, with the solutions incubated with the rat tissues, high degrees of ChE inhibition occured, indicating production of compounds with high inhibitory activity. The organs effective to activate parathion were the liver and kidney. The muscle and brain were inactive. These results agreed with those obtained by Gage[5] and Metcalf & March[8] with the slices of the rat liver.

Table 1. Activation of parathion by the slices of several organs of rat.

Addition	Per cent cholinesterase inhibition (%)	Increased per cent inhibition due to addition
Nil	5.5	
Liver-p-s*	+14.5	
Liver	97.0	91.5
Kidney	97.0	91.5
Muscle of hind-leg	15.0	9.5
Brain	4.0	+1.5

Reaction: Parathion (0.3 ml of 4×10^{-5}M) was added to the slices (0.7g) in a solution consisting of 2.6 ml of Krebs-Ringer solution (pH 7.4) and 0.1 ml of 3×10^{-1}M glucose in the presence of oxygen at 37°C.

* This experiment was run by adding parathion from the side arm at the end of the incubation.

Production of anticholinesterase substance from parathion by rat liver homogenate: Since homogenate is a heterogenous system, an attempt was made to localized activity in the subcellular fractionation of the homogenate. Davison[2] has reported that neither the cells and nuclei nor the mitochondria in the homogenate of rat liver activated schradan or parathion, and that the major part of the activity remained in the supernatant and microsome. The microsome fraction was further separated into microsome and a clear supernatant, but neither fraction was as efficient alone as the two fractions together. Moreover, he found that addition of nicotineamide was necessary for the conversion of parathion, which was considerably enhanced if NAD was added.

Activation of parathion by rat liver homogenate was studied by the same method as described by

Table 2. Activation of parathion by rat liver homogenate.

Addition	Per cent cholinesterase inhibition (%)	Increased per cent inhibition due to addition	N mg/0.1064
Nil	6.0		
Mitochondria			
cofactor+NAD (O_2)*	6.1	0.1	12
cofactor+NAD (air)**	6.1	0.1	12
Microsome			
cofactor+NAD (O_2)	97.0	91.0	2.9
cofactor+NAD (air)	98.0	92.0	1
cofactor+NADH (air)	97.0	91.0	1
Supernatant			
cofactor+NAD (air)	57.0	51.0	4.5

Each 3 ml of incubation mixture contained 2.5 ml of homogenate and the indicated concentrations of: 4×10^{-6}M parathion, 1.7×10^{-4}M nicotineamide-adenine nucleotide and 8×10^{-2}M nicotineamide. Incubation: 2 hours, at 37°C.

* In the presence of oxygen.

** In the presence of air.

Davison.[2] It was found that the mitochondria did not activate parathion, although the mitochondira did oxidize several compounds of the TCA cycle which were added to the medium. The major part of the activity remained in the washed microsome and supernatant after removal of mitochondria. A fraction more effective for activation was the washed microsome (Table 2). The essential additives were NAD, but the addition of NADH to microsome was also effective. O'Brien[11] showed that to activate parathion NADH was more effective than NAD in the washed microsome preparation of rat liver homogenate, and that using a system containing washed microsome, NADH, magnesium and nicotineamide, the following phosphorothionates were activated;

Table 3. Activation of parathion by whole homogenate of rice stem borer larvae.

Addition	Per cent cholinesterase inhibition (%)	Increase per cent inhibition due to addition	N mg/0.1064
Nil	6.5		
Mitochondria			
cofactor+NAD-p-s (air)*	0	+6.5	1.13
cofactor+NAD (air)	2.0	+4.5	1.13
cofactor+NAD (O_2)	1.4	+5.1	1.13
cofactor+NADP (O_2)	2.0	+4.5	1.44
Microsome			
cofactor+NAD-p-s (air)*	0	+6.5	4.1
cofactor+NAD (air)	15.5	9.0	4.1
cofactor+NADP (air)	63.0	57.5	4.1
cofactor+NADP-p-s (air)*	0	6.5	5.8
cofactor+NADP (air)	92.0	85.5	5.8
Supernatant			
cofactor+NAD (air)	11.2	3.5	11.2

The incubation mixtures were the same as shown in Table 2. *After incubating the reaction mixture for 2 hours, parathion was added from the side arm.

parathion, Co-Ral, malathion, Guthion, EPN, diazinon, Potasan, Ronnel, and the thiono analog of Tetram. This observation was in agreement with the results as shown in Table 2. However, it remains to be explored whether the essential cofactor for activation of ethyl parathion is NAD or NADH.

Production of anticholinesterase substance from parathion by insect homogenate: Although tissues of the rat were not equally effective to activate parathion in insect, almost all tissues converted parathion to paraoxon.[8] Because of small size of the rice stem borer, homogenate of the whole body was used. The results are given in Table 3. The activation system of parathion was found in the washed microsome as in the case of mammal. NAD was not effective as cofactor when added to mitochondria, microsome and supernatant. On the other hand, NADP was necessary for the activation of parathion in microsome. NADP had no effect in mitochondria, although several substrates of the TCA cycle were oxidized in them. These results are consistent with those of activation of Schradan and Guthion by insect homogenate mentioned above.[4,9] However, it remaines to be seen whether the essential cofactor in parathion conversion is NADP or NADPH. It is suggested that the activating systems are different between insect and mammal in that they require different cofactors.

Demonstration of active anticholinesterase substances by column and paper chromatography: Warburg flasks containing 4×10^{-5}M and 4×10^{-6}M purified parathion and the washed microsome prepared from homogenates of the rat liver and the borer larvae with cofactors were incubated at 37°C in the presence of air for two hours. In mammal, NAD was used as cofactor and in insect NADP was used. Control flasks without the microsome were also run. At the end of incubation period, the contents of each flask were extracted with chloroform. The chloroform extracts were evaporated in vacuo. The residue was dissolved in *n*-hexane-benzen(85 : 15) solution for silicic acid column chromatography. On the other hand, the residue of the chloroform extracts was partitioned between acetonitrile and *n*-hexane for the elimination of fats and waxes.[6] The acetonitrile layer containing parathion and its activated product was separated and then evaporated. The residue was dissolved in methyl alcohol for paper chromatography. The sample solution was put in the column. Each fraction was examined by Averell-Norris method and anticholinesterase assay. By Averell-Norris method, two peaks were found, one of them being identical with parathion and other with paraoxon. By anticholinesterase assay, the degree of enzymatic inhibition was high at the position of paraoxon, whereas no inhibition occurred for the other fraction (Fig. 1.) The sample solutions

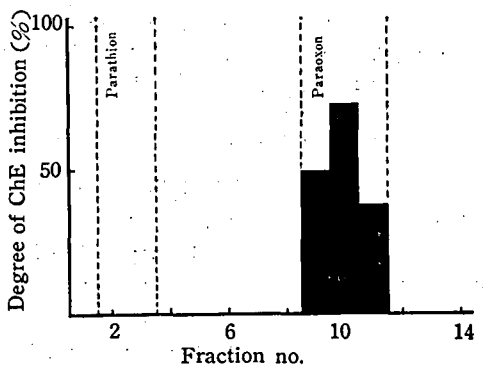


Fig. 1. Column chromatography on silic acid of the activation product by parathion in the washed microsome of the homogenates of rat liver and rice stem borer larva. Stationary phase: methanol-water(9:1); Mobile phase: benzene-*n*-hexane(I5:85).

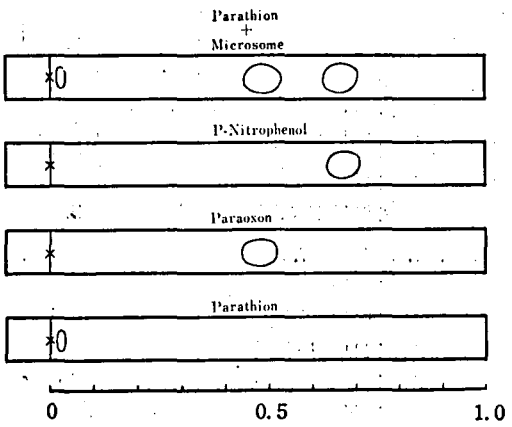


Fig. 2. Reversed phase paper chromatography of activation product in the washed microsome of the homogenates of rat liver and rice stem borer larva. Solvent system: chloroform-ethanol-acetone-water (0.5 : 1 : 1 : 4)/40% mineral oil.

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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