NOTES ON THE DECOMPOSITION OF PARATHION IN LIVING ORGANISMS*

I. PARATHION-SPLITTING ACTIVITY OF BLOOD SERUM AND SERUM CHOLINESTERASE

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Parathion, diethyl-p-nitrophenylthiophosphate, was first introduced into this country in 1952 as an insecticide to be used in fields for the elimination of rice stem borer (Chilo suppressalis Walker). Although parathion is highly effective against rice stem borer and has contributed a great deal to the increased yield of rice, its high toxicity has since been a grave concern of the authorities of public health and agriculture. The reports of poinsoning accidents among farmers due to careless handling of this drug is amounting to a considerable number.[1-4]

Contact with parathion causes a series of clinical manifestations of poisoning such as nausea, hypersalivation, tachycardia, convulsion and other symptoms which are directly related to the overexcitation of the cholinergic nervous system as a result of the inhibition of cholinesterase.[5-9] It has been well known that the parathion absorbed into the organism is decomposed and excreted in the urine. Para-nitrophenol is one of such endproducts.[5-10]

In early days of our animal experiments with parathion, it was conceived that this substance, being an organic phosphorus compound, might be split by a phosphoesterase. In order to elucidate this hypothesis, a following test was made. A small amount of an emulsified preparation of parathion was diluted with sodium carbonate-sodium bicarbonate buffer of pH 10 and then mixed with an aliquot of human serum. The mixture was incubated at 37°C and compared with a control mixture which lacked serum but was treated similarly. The test revealed a stronger yellow coloration in the sample mixture, indicating greater release of p-nitrophenol from parathion, than in the control. Evidience was thus provided for the presence of a parathion-splitting substance in the human serum. Therefore, further investigations were undertaken with resultant development of a procedure for the assay of such parathion-splitting activity in the serum.

Several observations[5-13] have been published on the alteration of serum cholinesterase activity and the urinary para-nitrophenol excretion in parathion poisoning, and their results, particularly of serum cholinesterase, have been applied to the diagnosis of intoxication of this inseticide.

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Since the parathion-splitting activity of the serum was expected to play an important role in the detoxication of the drug, a series of observations was made with dogs to follow the changes of such activities in the serum and their relations to serum cholinesterase and the urinary para-nitrophenol.

In addition, a similar study was conducted with diethylthiophosphate which was thought to be a transient product in the decomposition process of parathion and a toxic substance because of its still organic nature. The results of such studies will be reported herein.

MATERIALS AND METHODS

Twelve female dogs of 5 to 13 kg were divided into three groups, four in each. The dogs in each group were housed individually in cages and given 0, 0.5, 3.0 and 18.0 mg. of parathion per Kg body weight respectively by a single subcutaneous injection. Parathion was made injectable by emulsifuing it with a mixture of 1 part of toluene 1 part of Solpol and 48 parts of water in such a way that 1 to 1.5 ml contained the desired amounts of parathion. The animals receiving 0 mg., i.e., the emulsifying solvent only, served as the control.

Samples of blood, 6 to 7 ml were withdrawn from the dogs by cardiac puncture before, and 2 hours, 10 hours and 24 hours after the injection. From the second day through the seventh day the blood was taken once a day, and from the second week to the fourth week once every two days.

The urine was collected in a container through a funnel attached to the cage bottom. Urine samples were collected at 10 hours, 24 hours, 2 days 3 days...... and so forth after the injection of parathion.

For the purpose of examining the toxicity of diethylthiophosphate a very similar design of experiment was used with additional 12 female dogs. Varying amounts of potassium 0,0′-diethylthio phosphate (melting point: 196.5-197°C), namely its 0.0, 0.357, 2.144 and 12.870 mg per kg which were equivalent to 0.0, 0.5, 3.0 and 18.0 mg of parathion, injected.

I. Determination of parathion-splitting activity of blood serum.

Reagent.

1) Bicarbonate-carbonate buffer solution (pH 9.37). One-tenth molar solutions of sodium bicarbonate ($NaHCO_3$) and of sodium carbonate (Na_2CO_3) are prepared with C. P. grade reagents. The sodium bicarbonate solution and the sodium carbonate solution are mixed in the proportion of 3 to 1 to get the buffer solution of pH 9.37.

2) Parathion emulsion. One-fourth ml of the emulsified preparation of parathion of Fujikagaku Factory (47 per cent) is made up to 5.0 ml with ditilled water.

3) 5N-HCI. Concentrated hydrochloric acid (12 N, specific gravity 1.180 or more) is diluted 2.4 times with distilled water.

4) 0.7N-NaOH. 2.8 g/dl aqueous solution is prepared with Merck's C.P. grade sodium hydroxide.

5) Ethyl ether. C.P. grade ethyl ether is used.

6) Para-nitrophenol solution (lmg/ml). One g of p-nitrophenol (Merck) is dissolved in 1000 ml of 0.7 N-NaOH.

Procedure

Aliquots of 0.2 ml of the blood serum are introduced into test tubes A and B. Tube B is immersed in a boiling water bath for 8 minutes to inactivate the parathion-splitting activity of the serum. At the end of the specified time the tube is removed from the water bath and cooled in running tap water. One ml of buffer solution and 0.5 ml of parathion emulsion are added to tubes A and B, mixed and incubated at 37°C for an hour. Then the mixtures in both tubes are made acid to litmus paper with 5 N hydrochloric acid, and extracted with 5 ml of ethyl ether three times. Para-nitrophenol split from parathion (tube A) and p-nitrophenol produced by the simple alkaline decomposition of parathion (tube A and tube B) move to the ether layers. Ether-phase is pooled separately for tube A and tube B. The ether extract is shaken with 5.0ml of 0.7N-NaOH. (Para-nitrophenol passes into the sodium hydroxide layer, and yields yellow coloration.) After the separation of two layers, the upper layer (ether) is collected and filtered with a filter paper (Toyo-roshi No. 7). Two ml of the filtrate is diluted with 5 ml of distilled water for spectrophotometry (Shimadzu QB 50). The absorbances a and b of the diluted filtrates of tube A and tube B are measured at 397 mμ in a cell with 1.0 cm optical path.

Construction of calibration curve.

Five ml of 1 mg/ml p-nitrophenol solution is made up to 500 ml with 0.7 N-NaOH to prepare the working standard solution which has 10 γ of p-nitrophenol per ml. This standard solution is subjected to serial two-fold dilutions with 0.7 N-NaOH to obtain 5.0, 2.5, 1.25 and 0.63 γ per ml p-nitrophenol solution. The standard working solution and its dilutions are measured for their absorbances at 397 mμ. A calibration curve is constructed by taking the absorbances on the ordinate and the concentrations of p-nitrophenol on the abscissa. The calibration curve forms a straight line which passes the origin of the co-ordinates. The p-nitrophenol concentration α and β (γ/ml) of the diluted filtrates of tube A and B are read by comparing the absorbances a and b with the calibration curve.

Calculation

The parathion splitting activity of the serum is calculated from the following

equation.

$$\text{Parathion-splitting activity} = 87.5 \times (\alpha-\beta)\,\gamma/\text{ml}$$

II. Determination of serum cholinesterase activity.

The activity of serum cholinesterase was estimated by Takahashi-Shibata's phenol red comparator method.[14-15]

III. Determination of urinary p-nitorophenol.

Lawford-Harvey's method[1,3,16] was employed for the determination of p-nitrophenol in urine. The procedure is outlined as follows:

Ten ml of urine is hydrolysed with 5 ml of concentrated hydrochloric acid in a boiling water bath for an hour to decompose the conjugated p-nitrophenol. The hydrolysate which now has the free p-nitorophenol is extracted with 10ml of acetic ether while shaking. The acetic ether layer is separated and collected. Ten ml of 2 N ammonia are added to it and shaken to shift p-nitrophenol into ammoniacal aqueous layer. Aliquots of 5 ml of ammoniacal aqueous phase are transferred into centrifuge tubes A and B. To tube A are added 0.5 ml of 1.0 per cent o-cresol solution and 0.5 g of zinc powder, while 0.5 g of zinc powder only is introduced to tube B. The tubes are shaken and centrifuged to get a clear supernatant. The supernatant of tube A is colored blue because of a substance which has been produced by the coupling of o-cresol with reduced p-nitrophenol (p-nitorophenol is reduced by the zinc powder). The absorbances a and b of the supernatants of tubes A and B are measured at 620 mμ with a photoelectric colorimeter. The concentration of p-nitorophenol in the ammoniacal layer is read by comparing the difference of the absorbances (a–b) with a calibration curve which has been constructed by the same treatment of coloration applied to the standard p-nitrophenol solution (2.0, 5.0, 10.0 γ/ml p-nitrophenol solution in 2 N ammonia). The concentration of p-nitrophenol in urine is exactly the same as the p-nitrophenol concentration in the ammoniacal supernatant.

RESULTS

Twenty-four dogs which were used in our experiment varied in their parathion-splitting activity of serum within the range of from 105 to 350 γ/ml before the injection of parathion.

Injections of parathion in the doses of 0.5 mg and 18.0 mg per Kg induced no appreciable fluctuation in the serum parathion-splitting activity, remaining at almost constant levels of activity throughout the period of observation. The para thion-splitting activity of the animals which received the injection fell within $\pm$ 10 per cent levels around the average of the control animals. However, the dogs injected with 3.0 mg per Kg of parathion were different and had slightly

higher activities than the controls (Figure 1). A similar behavior of serum parathion-splitting activity was also observed with the potassium diethylthiophosphate (Figure 2).

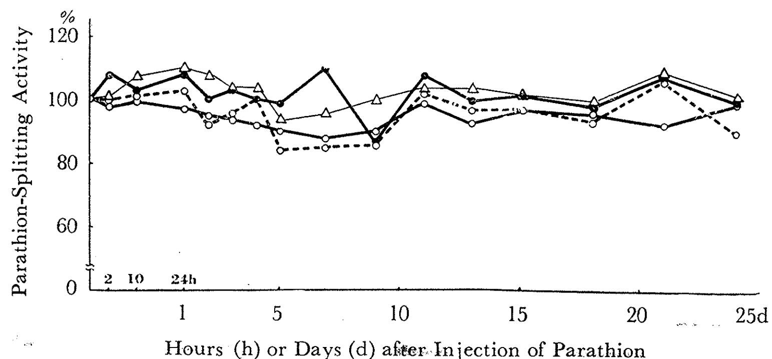

Fig. 1. Vicissitude of serum parathion-splitting activity in parathion-poisoned dogs. ○……○: Control, △——△: 0.5 mg per Kg, ●——●: 3 mg per Kg, ○——○: 18 mg per Kg.

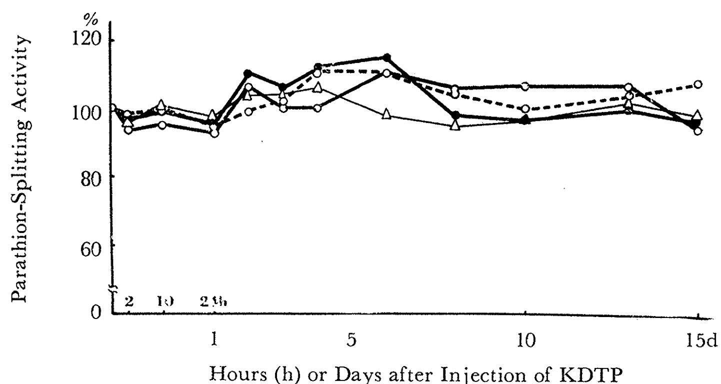

Fig. 2. Vicissitude of serum parathion-splitting activity in dogs injected with potassium diethylthiophosphate. ○……○: Control, △——△: 0.357 mg, ●——●: 2.144 mg, ○——○: 12.870 mg, KDTP.

In marked contrast to the serum parathion-splitting activity, both serum cholinesterase activity and urinary excretion of p-nitrophenol exhibited striking changes in parathion-poisoned dogs as shown in Figure 3. Nevertheless, potassium diethylthiophosphate exerted no influence on the activity of serum cholinesterase. There was neither inhibition of serum cholinesterase nor any sign of intoxication among the dogs injected with potassium diethylthiophosphate.

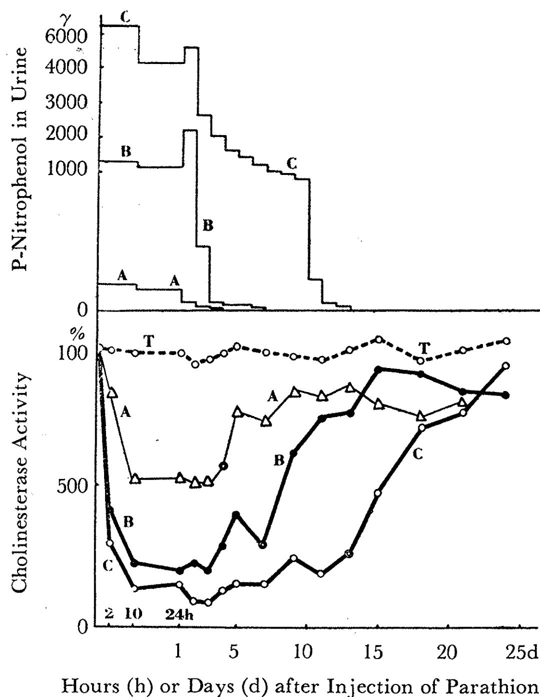

Fig. 3. Vicissitude of serum cholinesterase activity (bottom) and urinary excretion of p-nitrophenol (top) in parathion-poisoned dogs. AA: 0.5mg, BB: 3.0mg, CC: 18mg per Kg parathion, and TT: Control.

DISCUSSION

It will be apparent from the experimental results presented herein that the serum of the dog definitely contains a substance which is capable of promoting the decomposition of parathion. Similar parathion-splitting activity was also demonstrated in the human blood serum. The substance exhibits its maximum activity at 37°C, and is inactivated by heat over the temperature of 58°C (Figure 4). Its optimum pH is 8.6 to 8.8 (Figure 5). The amount of p-nitorophenol released from parathion increases in a linear proportion to the volume of serum added to the substrate (Figure 6). Excessive amounts of parathion (more than two times the amount specified in the procedure of determination) causes a reduction of parathion-splitting activity of the serum. Nickel ion (Ni^{++}) exerts a strong inhibition on parathion-splitting. As low as 2×10^{-2} Mol per liter of nickel ion reduces the activity to the level of 4 to 8 per cent of the control specimen. The decomposition of parathion is enhanced by the addition of sodium thiosulfate, azan and so forth (Table I). All these properties described above are interpreted as the evidence that the parathion-splitting substance in serum is an enzyme, because the substance meets the necessary requirements as an enzyme (Table II.)

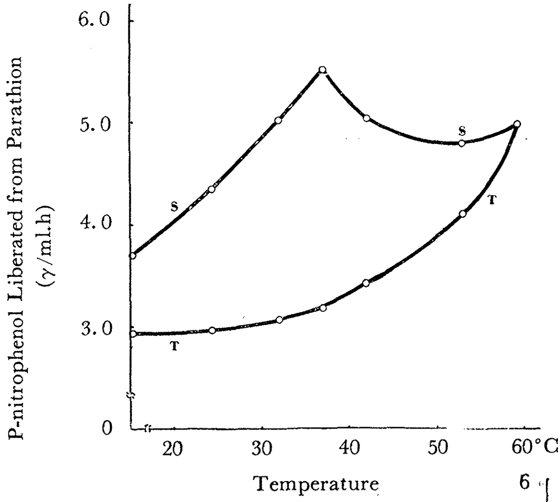

Fig. 4. Optimum temperature of serum parathion-splitting activity. SS: Sample tube containing blood serum in addition to substrate. TT: Control tube lacking blood serum.

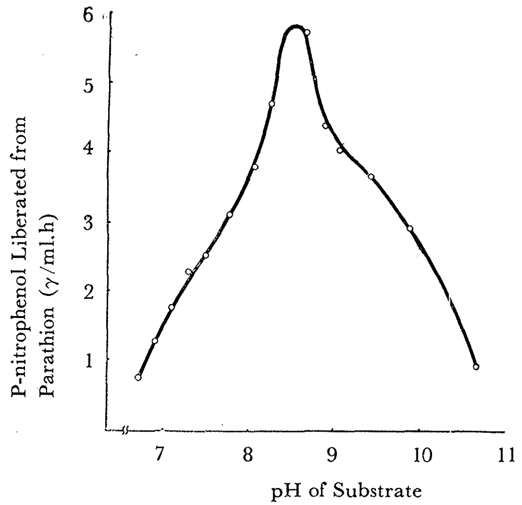

Fig. 5. Optimum pH of serum parathion-splitting substance.

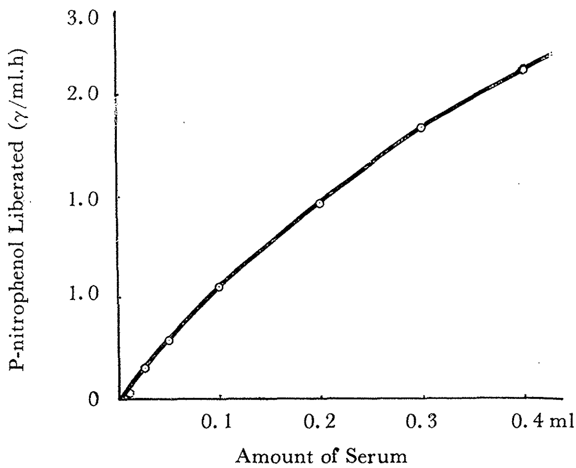

Fig. 6. P-nitrophenol liberated from parathion in relation to the amount of serum used.

Table I

Activators of serum parathion-splitting substance and their activation rates (%)

Activators	Dilution of the activators 1×	5×	25×	125×
50 % Glucose	7.5	5.0	0.9	0.1
20 % Fructose	9.7	8.0	7.0	6.2
Azan	4.6	3.7	3.6	—
10 % Sodium thiosulfate	15.8	11.1	8.8	8.5
Banthionin	5.8	6.7	3.6	—

Tabel II

Properties of serum parathion-splitting substance as compared with the requirements of enzyme (hydrolase).

Properties required to be an enzyme	Parathion-splitting substance
1. Thermolability	Destroyed by heat over the temperature of 58 °C
2. Specificty to substrate	No substance other than parathion has been decomposed
3. Inhibitors (sensitivity to heavy metal ions)	Inhibited by nickel ion
4. Activators	Glucose. fructose, azan and thiosulfate
5. Optimum temperature	37°C
6. Optimum pH	8.6—8.8
7. Optimum concentration of substrate	55 fold dilution of Fujikagaku's emulsified parathion

Serum cholinesterase has hitherto been regarded as the enzyme which is most closely related to parathion poisoning. If the parathion-splitting substance in serum is an enzyme as discussed, the identification of the substance with the serum cholinesterase ought to be considered. However, it is very unlikely because, as stated in the preceding section, the serum parathion-splitting activity remains unchanged in parathion poisoning in spite of the marked decrease in the serum cholinesterase activity. Absence of correlation between the parathion-splitting substance and cholinesterase invalidates their identity.

Summary and Conclusion

1. An enzyme-like substance has been discovered in the canine blood serum which decomposes parathion (diethyl p-nitrophenylthiophosphate).

2. This substance is not identical with the serum cholinesterase. The parathion-splitting activity of the serum remained unchanged in dogs following an injection

of parathion in emulsion, neither was there any correlation between the serum parathion-splitting activity and the urinary p-nitrophenol.

3. Potassium diethylthiophosphate did not interfere with the serum cholinesterase activity, and therefore may be a nontoxic substance.

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