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11. Masayasu Kimura, Toshiji Igarashi, and Sadao Iwashita*1 : Molecular Pharmacological Studies on Drug-Receptor Complexes System in Drug Action. II.[1] The Mode of Action of Parathion on an Acetylcholine Receptor.*2

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In a preceding paper,[1] it was reported that parathion, one of organophosphates, may be considered as a pharmacological antagonist against acetylcholine (ACh). Erdmann, *et al.*[2] also pointed out that the paralytic effects of parathion on intestine seemed to be produced by its direct action on the smooth muscle. Other experiments[3] on the effects of the phosphines upon synaptic conduction of the roach suggested that the organophosphates can, besides inhibiting cholinesterase (ChE), block an ACh receptor at high doses. However, very few studies have so far been made to clarify the relationship between ACh and parathion and also the blocking action of parathion against ACh. The present paper may be the first dealing with the elucidation of the mode of action of parathion on ACh receptor.

In the present paper, some observation was made on the dose inhibitory response curve of parathion and 2-pyridinealdoxime methiodide (PAM) in the isolated intestine of mice, and the results were analysed stochastically in order to study the antagonism of parathion and PAM against ACh, and then to compare it with the action of parathion against ChE. Furthermore, it was examined by Bürgi's rule how parathion, PAM and atropine interact with each other in smooth muscle, since it is the object of this paper to show that the inhibitory action of parathion can be produced by its combination with ACh receptor on smooth muscle.

Method and Experimental

Biological conditions and experimental methods were the same as described earlier,[4] and the intestinal segments of mice used were immersed for 3 min. in Tyrode solution containing the antagonist prior to the administration of ACh. After the antagonist was washed out, the next administration of drugs was made after a resting period of 20 min. In this experiment, parathion was used in two forms, namely an emulsion containing 46.6% of parathion and a solution of an industrially pure sample dissolved in Gum. Arab. The results of the dose inhibitory response curve were analysed stochastically by the theoretical method of which a detailed description was given in the previous papers.[5~7] The experimental method used for ChE was also the same as in the preceding paper.[1]

Experimental design for estimating the effect of interaction by antagonists each other is as follows: When each of two antagonists A_1 and A_2 produces the same inhibitory effect against ACh, a half dose of the total mixture of A_1 and A_2 can also produce a similar result if both two attack the same point of action. If each antagonist attacks a different point of action, the effect caused by the mixture will be stronger than that of a single antagonist, and its inhibition curve will be shifted to the left. As an adequate example of the above design, the combination of atropine (Atr) and its methobromide

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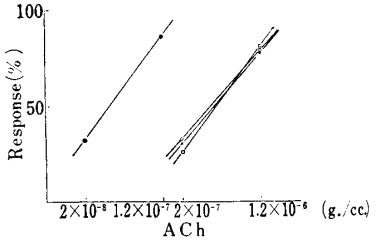
1) Part I. M. Kimura : This Bulletin, **11**, 44(1963).
2) W.D. Erdmann, D. Heye : Arch. exptl. Pathol. Pharmakol., **232**, 507 (1958).
3) K.D. Roeder, N.K. Kennedy : J. Pharmacol. Exptl. Therap., **114**, 211 (1955).
4) K. Takagi, M. Kimura : This Bulletin, **4**, 444 (1956).
5) K. Takagi, M. Kimura : *Ibid.*, **4**, 449 (1956).
6) K. Takagi, *et al.* : Yakugaku Zasshi, **76**, 1191 (1956).
7) M. Kimura : "Suikeigaku-no-Kagaku-to-Seibutsugaku-e-no-Ohyō III," in Kagaku-no-Ryoiki, Zokan, **36**, 37 (1956), Nankōdō, Tokyo.

Table I. The Results of Trial Design for Determining the Point of Action of Drugs (ACh Effect in the Presence of Antagonist; % for the Maximum Contraction of 10^{-4} g./cc. of ACh)

ACh (g./cc.) / Antagonist	2.0×10^{-8}	1.2×10^{-7}	2.0×10^{-7}	1.2×10^{-6}
without	32.6	86.1		
Atropine (Atr)			32.2	78.1
Atr-MeBr			25.8	80.3
$\frac{\text{Atr}+\text{Atr-MeBr}}{2}$			31.8	77.5



⊙ ACh only
○ Atr
□ Atr-MeBr
× $\frac{\text{Atr}+\text{Atr-MeBr}}{2}$

Fig. 1. Synergistic Effect between Atropine (Atr) and Atr-MeBr at Acetylcholine Receptor

was selected since both attack the same point of action. The results of these tests are shown in Table I and Fig. 1.

Since the result shown in Fig. 1 is in agreement with the earlier observations,[8] the experimental design described above seems to be advantageous.

Results

I. Inhibitory Effect by Parathion upon Dose Response Curve of ACh

The effect of parathion upon shifting the dose response curve of ACh is given in Table II and Fig. 2.

Table II. Data of Dose Response Curve of ACh with Parathion (unit : %)

Parathion (M) / ACh (M)	0	1.37×10^{-7}	3.44×10^{-6}	1.37×10^{-5}
5.50×10^{-8}	13.0	16.1		
1.10×10^{-7}	30.2	24.0		
2.20×10^{-7}	62.6	40.6	12.6	
4.40×10^{-7}	84.4	69.5	26.4	
8.80×10^{-7}	90.2	82.1	52.6	
1.76×10^{-6}		93.3	64.1	
3.52×10^{-6}			73.8	
7.04×10^{-6}			87.1	
1.41×10^{-5}			94.8	
5.50×10^{-5}				26.3
1.38×10^{-4}				34.8
2.75×10^{-4}				47.0
5.50×10^{-4}	100	100	100	54.5

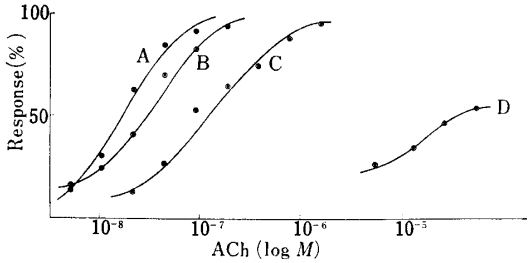


Fig. 2. Shift of the Dose Response Curve of ACh by Parathion

Parathion Doses (M);
B : 1.37×10^{-7}
C : 3.44×10^{-6}
D : 1.37×10^{-5}.

8) K. Takagi, M. Kimura : This Bulletin, 5, 440 (1957).

Table II shows the rates of contraction in the same intestinal muscle by various doses of ACh against the given doses of parathion (1.37×10^{-7}, 3.44×10^{-6}, and $1.37\times10^{-5}M$), and the number in the Table indicates the per cent ratio calculated from the mean value of 6 measurements. In Fig. 2 are plotted the dose response curves of ACh shifted by parathion. From these results, it was found that parathion inhibits ACh at least competitively at doses less than $3.44\times10^{-6}M$.

II. Dose Inhibitory Response Curve by Parathion against a given Dose of ACh

Using two sorts of parathion, the mode of action on an ACh receptor was observed. In Table III are shown the contraction ratio of the same intestinal muscle by given doses of ACh (e. g. 2.75×10^{-7}, or $5.5\times10^{-4}M$)[9] in the presence of various doses of parathion both in Gum. Arab. (P-G) and in emulsion (P-E). Figs. 3 and 4 show the curve and the logistic regression line obtained from the data in Table III.

Table III. Data of Dose Inhibition Response Curve by Two Forms of Parathion against a given Dose of ACh

ACh (M)	Parathion (M)	Repetition	Means (%) P-E	Means (%) P-G
2.75×10^{-7}	2.18×10^{-7}	10	68.9	
	5.44×10^{-7}	10	50.1	
	1.36×10^{-6}	10	30.5	90.8
	3.44×10^{-6}	10	17.0	73.6
	8.50×10^{-6}	10		48.6
	2.10×10^{-5}	10		26.7
5.50×10^{-4}	2.27×10^{-5}	10	72.7	
	3.44×10^{-5}	10	57.0	
	5.10×10^{-5}	10	41.3	
	7.65×10^{-5}	10	25.0	

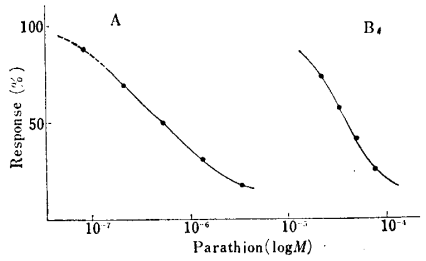


Fig. 3. Dose Inhibitory Response Curves by Parathion in Emulsion against a given Dose of ACh

Curve A : Atropine-like Inhibition against $2.75\times10^{-7}M$ ACh

Curve B : Papaverine-like Inhibition against $5.50\times10^{-4}M$ ACh

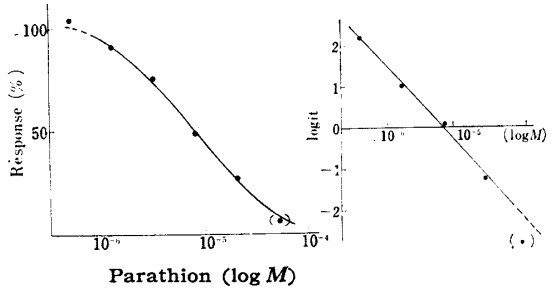


Fig. 4. Dose Inhibitory Response Curve by Parathion in Gum Arab. against a given Dose $2.75\times10^{-7}M$ of ACh, and its Logistic Line

In Table IV, are summarized the analysis of the variance of the data in Table III, and the estimation of slope of the logistic lines. In Table IV, the linearity of a logistic regression line of each curve was recognised significantly and the slope was estimated as 1 in both cases of P-G and P-E against ACh ($2.75\times10^{-7}M$) and as 1.5 against a higher dose ($5.5\times10^{-4}M$) of ACh.

9) K. Takagi, I. Takayanagi : This Bulletin, **9**, 580 (1958).

Table IV. Analysis of Variance of Data of Table III and Estimation of Slope of Logistic Regression Lines

ACh dose / Nature of Variance	d.f.	ACh $2.75\times10^{-7}M$ P-G mean square	ACh $2.75\times10^{-7}M$ P-E mean square	ACh $5.5\times10^{-4}M$ P-E mean square
Parathion	3	8702.0	5147.5	4205.4
Animals	9	944.0	124.8	6.3
Error	27	349.4	65.2	2.9
Linearity (D_0^2)		101.1	40.0	3.4
Slope "b"		− 1.20	− 0.87	− 1.69
$\sqrt{V(b)}$		0.098	0.054	0.032
Supposed value of b		− 1	− 1	− 1.5
Slope Test t_0 (t=4.30, d.f.=2)		2.04	2.34	2.81

III. Dose Inhibitory Response Curve by Parathion against ChE

This experiment was made in order to compare the mode of action of parathion against ChE with that against an ACh receptor. Warburg method was employed, by which parathion effects were measured after 50 min., using $1.1\times10^{-1}M$ ACh and 2×10^{-2} g./cc. crude serum ChE of guinea pig. The results are shown in Tables V, VI and Fig. 5. In Table VI, an indication of the linearity of the logistic line of parathion against ChE was observed significantly and the slope was estimated as 1 (see Fig. 5).

Table V. The Anti-ChE Effect of Parathion

Parathion Dose (M)	Repetition	Mean (%)
3.40×10^{-7}	6	5.5
1.02×10^{-6}	6	29.0
3.06×10^{-6}	6	46.2
9.18×10^{-6}	6	65.4
2.75×10^{-5}	6	80.3

Table VI. Analysis of Variance of the Data in Table V and Estimation of the Slope of the Logistic Regression Line

Nature of Variance	d.f.	Mean Square
Parathion Doses	4	5314.4
Repetition	5	75.5
Error	20	105.0
Linearity	3	253.2
Line Test F_0 (F^3_{20}=5.82)		2.41
Slope "b"		0.89
Supposed value of b		1
Slope Test t_0 (t=3.18, d.f.=3)		0.73

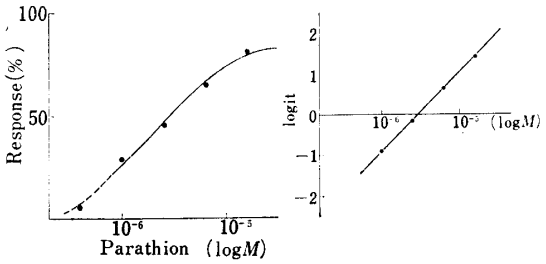


Fig. 5. Dose Inhibitory Response Curve by Parathion against ChE, and its Logistic Line; Warburg Method using Crude Serum ChE

IV. Dose Inhibitory Response Curve by PAM against a Constant Dose of ACh

With the same purpose as in the Experiment II, PAM was employed in this experiment. The effects of PAM against $2.75\times10^{-7}M$ ACh are shown in Tables VII, VIII and Fig. 6. Fig. 6 shows a curve obtained from the data in Table VII and its logistic

Table VII. Dose Inhibitory Response Curve of PAM against ACh

PAM (M)	Repetition	Mean (%)
2.0×10^{-5}	6	79.7
4.0×10^{-5}	6	57.2
8.0×10^{-5}	6	49.0
1.6×10^{-4}	6	31.4
3.2×10^{-4}	6	21.1

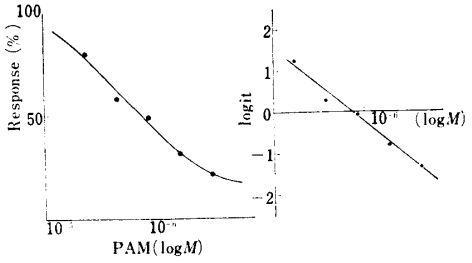


Fig. 6. Dose Inhibitory Response Curve by PAM against a given Dose $2.75\times10^{-7}M$ of ACh, and its Logistic Line

Table VIII. Analysis of Variance of Data in Table VII and Estimation of the Slope of the Logistic Line

Nature of Variance	d.f.	Mean Square
PAM Dose	4	3129.7
Animal	5	1207.0
Error	20	69.8
Linearity	3	159.0
Line Test $F_0(F^3_{20}=5.82)$	2.28	
Solpe "b"	− 0.89	
Supposed value of b	− 1	
Slope Test $t_0(t=3.18,$ d.f.$=3)$	0.83	

regression line. Table VIII shows the analysis of variance of data in Table VII, together with the calculation from which the slope of a curve of PAM is estimated. In Table VIII, a tendency of the linearity of the logistic line of PAM effect against ACh was recognised significantly and its slope was estimated as 1.

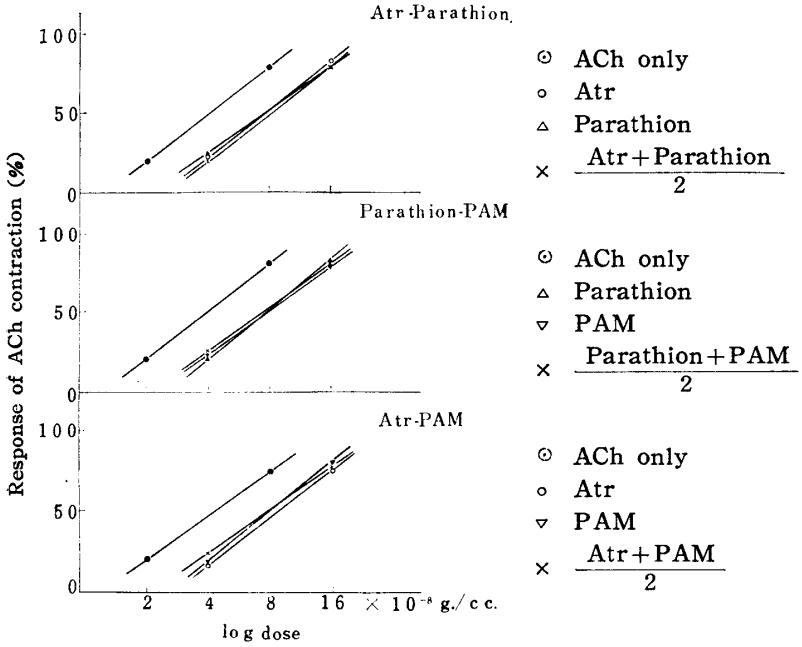


Fig. 7. Interaction Effects between two Antagonists of Atr, Parathion, and PAM upon ACh Receptor

V. Interaction between Two Antagonists, consisted of Atropine, Parathion, and PAM upon ACh Receptor

According to the experimental design described above, the relation of three pairs, atropine and parathion, parathion and PAM, and atropine and PAM, was examined. The doses of each antagonist were adjusted so as to make the response curve of ACh (2×10^{-8} g./cc. and 8×10^{-4} g./cc.) without inhibitor to shift to the inhibited curve against ACh (4×10^{-8} g./cc. and 1.6×10^{-7} g./cc.). The shift of the inhibited curve of ACh was caused by half doses of each two antagonists. The results are shown in Fig. 7. From Fig. 7, it is evident that all of three pairs produce the synergistic effect of additive relationship.

Discussion and Conclusions

The mode of action of parathion must be argued, first of all, on the basis of the results of the experiment I shown in Table II and Fig. 2. These observations apparently indicate that the dose response curve of ACh is shifted to the right hand in parallel with the increase of the doses of parathion, but that is hardly the case with parathion at doses greater than $10^{-5}M$. From these observations the following deductions may be drawn : at doses less than $5\times10^{-6}M$, parathion can be at least a competitive antagonist against ACh, but can be either a competitive or a non-competitive one when the dose becomes greater than $10^{-5}M$.

Secondly, with regard to both the competitive and non-competitive inhibitions of parathion against ACh, their inhibitory response curves are obtained in the experiment II, as shown in Table III and Figs. 3 and 4. All of the logistic lines of these curves indicated a straight line relationship from a stand-point of statistical analysis. This seems possible to express the antagonistic action of parathion by the Guddum's equation.[10] From these calculations the difference of values between the slope "b" and the theoretical value 1 was thus found no significance in the case of the competitive inhibition. Concerning this value of the slope of competitive response curve, there was no difference observed between the results using parathion in Gum. Arab. and parathion in emulsion. The above results suggest that one parathion molecule antagonizes one ACh molecule at ACh receptor.

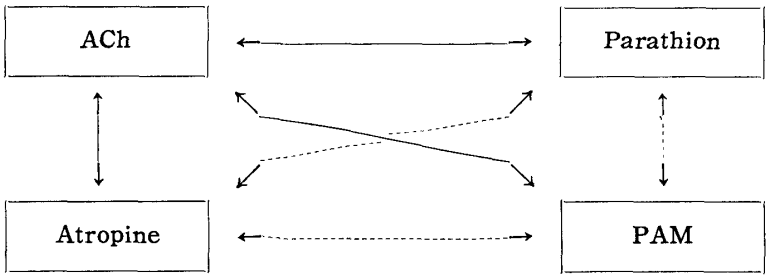
The next attempt was to observe the mode of action of parathion against ChE. Although an extensive discussion of this problem was described by Inoki,[11] the same observations were repeated for the purpose of comparing the attacks against ChE with ACh receptor by parathion. The result indicated that ChE and parathion react with each other in the process of the hydrolytic action of ChE. Therefore, even if parathion acts on ChE as well as on ACh receptor at the synapsis of the smooth muscle, the mode of action of parathion upon an ACh receptor will not be influenced by ChE. This fact seems to be interesting and invaluable in resolving the problem on the relationship between ChE and ACh receptors.

Furthermore, combination of PAM with ChE as the antidote of organophosphorous compounds clearly demonstrated that PAM attacks not only ChE, but also an ACh receptor. From the results of the experiment IV, it is evident that one PAM molecule, as well as in the case of parathion, antagonises one ACh molecule at an ACh receptor.

Finally, the site of action of parathion on the smooth muscle has been studied in detail. According to the results that parathion blocks competitively ACh, it can be expected to have an atropine-like action, although its effect is weaker than atropine

10) J.H. Gaddum : J. Physiol., **89**, 7 (1937).
11) R. Inoki : Folia Pharmacol. Japon., **55**, 1 (1959).

itself. Experiment V on the synergistic effect of atropine with parathion indicated that the combined effect of atropine and parathion is in the relation of addition, which was established by using PAM as an intermediate, for example, by examining the effects of the combination between atropine and PAM, or parathion and PAM. These relationships are illustrated by the following schematic form.



Scheme. Relationships between Inhibitors at ACh receptor
Additional Synergism : ←·····→; Antagonism : ←——→

From these expriments, it may be concluded that parathion attacks the same site of action as atropine, that is to say, an ACh receptor.

Summary

In order to study the mode of action of parathion upon acetylcholine (ACh) receptor, its inhibitory effect against ACh was examined with Magnus method using the isolated intestine of mice. These experiments gave following results :

1) From the result of a shift effect of parathion on the dose response curve of ACh, the mode of action of parathion was shown to be a competitive inhibition against ACh at doses less than $5\times10^{-6}M$, but to be a non-competitive inhibition at doses greater than about $10^{-5}M$.

2) In the com petitive inhibitiory effect, the estimation of the reaction order n was not significantly different from a supposed value 1, while the n was estimated as 1.5 in the case of the non-competitive inhibition. From this fact, it was concluded that one parathion molecule antagonises one ACh molecule at ACh receptor.

3) Likewise, the reaction order n of PAM against ACh was examined by Magnus method and ACh against ChE by Warburg method, and both were estimated as 1.

4) Using parathion, PAM and atropine, the interactions between two of them to an ACh receptor were demonstrated, and all of the three pairs were shown to have relationship of additive synergism. Therefore, it was confirmed that the point of action of parathion as well as atropine is the ACh receptor.

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