Authors are indebted to Mr. A. Nomura for his skillful performance in enzymatic analyses. A part of this study was supported by the Grant-in-Aid for Scientific Research from the Ministry of Education, which is gratefully acknowledged.

Summary

Phosphorylation of adenosine with morpholino phosphorodichloridate, P^1-diphenyl P^2-morpholino pyrophosphorochloridate and 2,6-lupetidyl phosphorodichloridate in the presence of boric acid was performed in DMF solution. It was observed that the ratio of resulting 5′-monophosphate to 2′- + 3′-monophosphate was in the range of 2.7~5.5, which indicated the protection of borate complex on 2′- and 3′-hydroxyl group of adenosine against the attack of the phosphorylating agent.

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21. Masayasu Kimura*1 : Molecular Pharmacological Studies on Drug-Receptor Complexes System in Drug Action. III.[1] The Site of Action of Organophosphate Group on the Acetylcholine Receptor Surface.*2

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In a preceding paper,[1] the authors described that the blocking effect of parathion against acetylcholine (ACh) might be produced by combining a molecule of parathion with an ACh receptor. According to this fact, the phosphate group of the parathion must have an affinity for either of the esteratic site or the anionic site of ACh receptor, of which the active surface is thought to be similar to that of cholinesterase (ChE), in order to make a parathion molecule fit into an ACh receptor.

On the other hand, experiments[2~4] have brought forward considerable evidences to support that ChE is phosphorylated at the esteratic site by organophosphate. Therefore, to compare the binding pattern of the parathion-ACh receptor complex with that of parathion-ChE complex will contribute to find a clue whether both ACh receptor and ChE have a similarity of feature or not.

First of all in the present paper, an experimental design for discriminating the site of action of antagonists was established, and the site of action of organophosphate group on ACh receptor surface was stochastically able to be estimated. Secondly the estimation was recognized by the other experiment on the competition of phosphate derivatives with pyridine aldoxime methiodide (PAM) by the steric hindrance of their chemical structure.

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*1 5-Okuda, Toyama (木村正康).

*2 This was published at the 33th meeting Japanese Pharmacological Society in Gifu. (May, 1960).

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3) I.B. Wilson : *Ibid.*, **199**, 113 (1952).
4) D.R. Davies, A.C. Green : Biochem. J., **63**, 529 (1956).

Method and Experimental Design

Biological condition and experimental method were the same as those described in the previous paper,[5] using the intestinal segments of mice by Magnus method.

An effectived modified method for determining the site of action of antagonist on ACh receptor surface $[R]$ was evolved from Kanazawa's paper[6] and Matsumoto's communication.[7] According to them, the inhibitory effect I of antagonist $[B]$ under a constant dose of ACh $[A]$ and antagonist $[C]$ is as follows:

$$I=\frac{[B]}{[B]+[G]} \qquad (1)$$

where, $$G=\frac{1+[A]/K_a+[C]/K_c}{1/K_b+[C]/K_b'\cdot K_c}=\frac{(1+[A]/K_a)}{1/K_b}\left\{1+\frac{[C]/K_c(1/K_b-1/K_b'-[A]/K_a\cdot K_b')}{(1+[A]/K_a)(1/K+[C]/K_b'\cdot K_c)}\right\}$$

under the K_a, K_b, K_c, and K_b' are the dissociation constants respectively of the next formulas (1)~(4). Expression I can be derived from the formula (5).

$$[A]+[R]=[AR] \qquad (1)$$
$$[B]+[R]=[BR] \qquad (2)$$
$$[C]+[R]=[CR] \qquad (3)$$
$$[B]+[CR]=[BCR] \qquad (4)$$
$$[R]+[AR]+[BR]+[CR]+[BCR]=1 \qquad (5)$$

Now, according to $K_b'=K_b(1+[A]/K_a)$ derived from $(1/K_b-1/K_b'-[A]/K_a\cdot K_b')$ in the expression G, the shifting of the curve can be classified into the following cases;

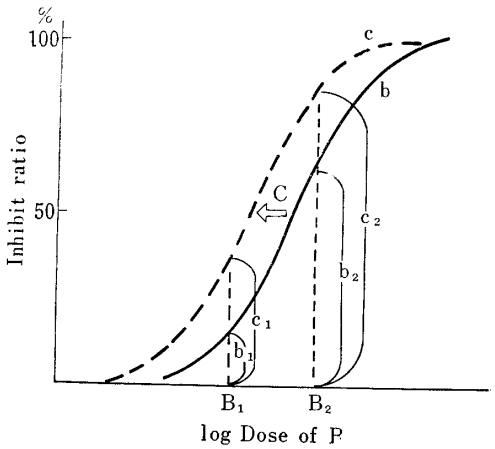
(a) if $K_b'\to\infty$, the curve will be shifting to the right. This effect means the complete association of two inhibitors with the same site.

(b) if $K_b'>K_b(1+[A]/K_a)$, the curve will be shifting to the right, too. This effect suggests the association of two inhibitors with the same site under interfering between them.

(c) if $K_b'=K_b(1+[A]/K_a)$, the curve will be not shifting.

(d) if $K_b'<K_b(1+[A]/K_a)$, the curve will be shifting to the left. This effect suggests the association of two inhibitor with the different site under interfering between them.

(e) if $K_b'=K_b$, the association of two inhibitors will the different site will be complete.



		Antagonist				
	ACh	B_1	B_2	C	B_1C	B_2C
Inhibit ratio	100	b_1	b_2	100	C_1	C_2

curve b : single antagonist inhibition
curve c : combined inhibition of B and C

Fig. 1. Scheme to Illustrate the Shifting Effect by Means of the Association of the Antagonist B with a Different Active Site from the Antagonist C in the Inhibitory Action against Acetylcholine

5) K. Takagi, M. Kimura : This Bulletin, **4**, 444 (1956).
6) T. Kanazawa : Folia Pharmacol. Japon., **53**, 207 (1957).
7) S. Matsumoto : A private communication to the authors (1959).

Thus, when the inhibitory ratio by one of antagonist (*e.g.* B) against a given dose of ACh as ordinate is plotted against the log of B as abscissa, a dose-inhibition curve of B to ACh can be given in Fig. 1. as the curve b.

If the constant dose of another antagonist (*e.g.* C) is applied in this antagonism, two cases will be indicated by the above formulas. (i) The original curve b, will be shifted to the left hand by C so that the new curve c may be drawn. Since the curve c is plotted with an inhibitory ratio of B against the response of the given dose of ACh, which is formerly inhibited by the constant dose of C, it can be estimated as the actual effects of B in the presence of C. This case indicates the association of B with a different active site from C. (ii) The other cases, where the curve c overlaps on or shifts to right hand from the curve b, indicate the association of B with the same active site as C. Whether the curve c overlaps on or shifts to the right hand from the curve b, depends upon the ratio of doses of C to B. Now, from the theoretical inhibition curve corresponding to the curve b, the calculated values for the curve c were plotted in Fig. 2 according to the various times larger than the dose of b as the substitute for C. Fig. 2 shows that the curve c overlaps on the curve b, if the dose of C is less than the dose of B caused 50% inhibition.

Thus, the alternative of these two cases can stochastically be estimated by the significant test of the factor "Preparation" in the analysis of variance. In the practical method, therefore, the plotting of the inhibitory curve is enough to be represented with two doses.

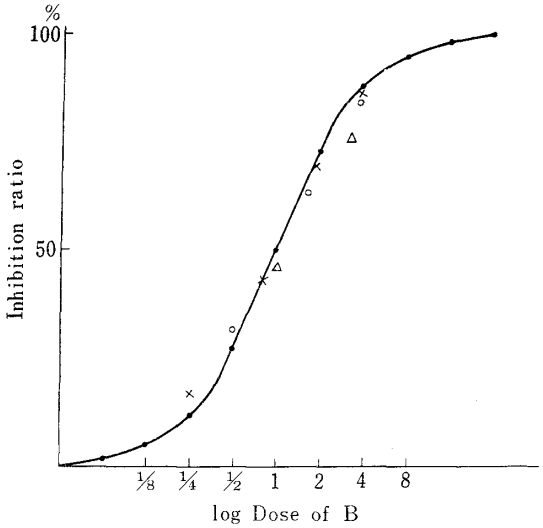


Fig. 2.

The plotted values for the curve c calculated from the theoretical inhibition curve (—●—) corresponding to the curve b in Fig. 1., in the case of that the doses of B assumed an inhibitor C are the same (△), half (○), quarter (×) times as much dose of B caused 50% inhibition.

Results

I. Trial Test for the Validity of the Experimental Design

In purpose to evidence the validity for the method of determination described above, observations were made on the following two preparations due to the different dose of atropine, for example, a combination of the two doses of atropine (8×10^{-10}, 24×10^{-10} g./ml.) as the antagonist B with the other given dose of atropine (8×10^{-10} g./ml.) as the antagonist C. The other pair is a combination between methylparathion (4.8×10^{-7}, 16×10^{-7} g./ml.) and parathion (2×10^{-7} g./ml.). The results are shown in Tables I, and II, of which the mean inhibitory ratio was calculated from assuming 100 as the contractive response in both cases of non antagonist and only antagonist C.

Table I. Data for Interaction between Two Doses of Atropine against Acetylcholine (5×10^{-8} g./ml.)

Atropine as B	—	8×10^{-10}	24×10^{-10}	—	8×10^{-10}	24×10^{-10}
Atropine as C	—	—	—	8×10^{-10}	8×10^{-10}	8×10^{-10}
Contractive response by ACh (mm.)	76.0	43.0	37.5	43.0	37.8	28.9
	69.9	51.5	35.0	51.5	35.2	34.0
	41.5	36.5	15.8	36.5	29.5	8.9
	55.1	44.5	27.0	44.5	38.5	10.9
	45.0	34.8	24.0	34.8	18.0	15.0
	71.1	51.0	31.5	51.0	18.7	32.3
	41.5	32.4	28.5	32.4	22.6	11.0
Mean inhibitory ratio (%)	0	24.8	51.3	0	28.0	57.9

Table II. Data for Interaction between Methylparathion and Parathion against Acetylcholine (5×10^{-8} g./ml.)

Methylparathion	—	4.8×10^{-7}	1.6×10^{-6}	—	4.8×10^{-7}	1.6×10^{-6}
Parathion	—	—	—	2×10^{-7}	2×10^{-7}	2×10^{-7}
Contractive response by ACh (mm.)	83.0	78.0	66.0	77.8	76.0	54.5
	65.8	51.1	33.5	54.5	50.5	29.0
	37.5	32.9	26.9	36.8	31.9	16.3
	38.9	37.5	25.0	31.5	27.8	21.7
	30.8	26.8	20.8	29.0	23.9	16.8
	45.9	42.5	17.5	42.5	33.2	17.0
	53.5	31.2	20.8	44.0	28.8	16.0
Mean inhibitory ratio (%)	0	15.4	39.1	0	15.6	47.0

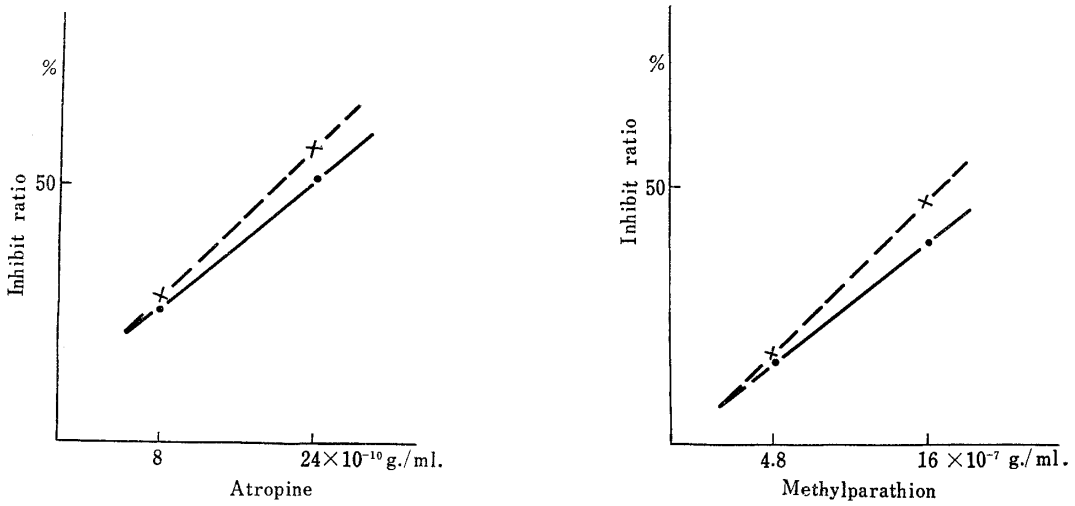


Fig. 3. The Effect of Interaction between Two Doses of Atropine (in the left hand), and between Methylparathion and Parathion (in the right hand)
The F-test was determined in p=0.05.

For these data, Table III gives the analysis of variance in order to determine the relation between two lines, which have been plotted in graphical form in Fig. 3.

Table III. Analysis of Variance for Data of Tables I and II

Combination of antagonists / Nature of variance	d.f.	Atropine two doses Mean square	Methylparathion and parathion Mean square
Preparation	1	179.53	113.20
Regression	1	5496.41	5324.04
Parallelism	1	74.58	104.53
Between doses	3	1916.84	1847.26
Between animals	3	125.52	994.64
Error	18	225.68	73.86
Test of parallelism F_0 ($F^1_{18}=4.41$)		0.33	1.42
Test of preparation F_0 ($F^1_{18}=4.41$)		0.80	1.53

From Table III, in both case of two pairs, it was seen that parallelism and preparation between two lines were not significantly different, that is to say, the shifting effect was hardly caused. Since this results show that the two preparations of atropine,

and a pair of methylparathion and parathion have respectively the same active site of ACh receptor surface, there is a sufficient evidence to support the validity of this experimental design.

II. Interaction of Parathion, Pyridine Aldoxime Methiodide, Atropine and Isopentyl Acetate upon Two Different Active Sites of Acetylcholine Receptor Surface

The experiments on the interaction of each one of four pairs; atropine and parathion, PAM and atropine, parathion and PAM, and parathion and isopentyl acetate yielded the following results :

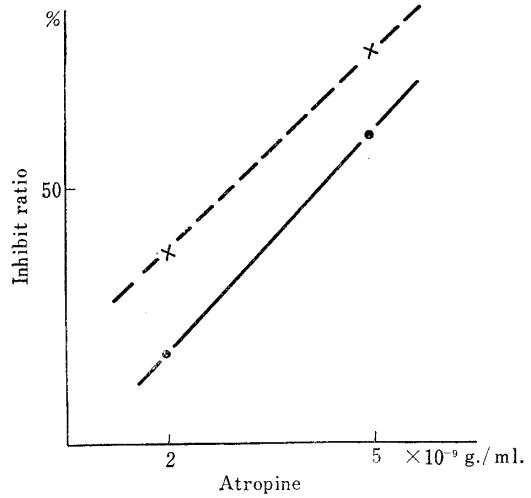
(a) Atropine and parathion

The experimental design of this pair consists of two doses 2×10^{-9}, 5×10^{-9} g./ml. of atropine against 3×10^{-7} g./ml. parathion, and the data were made from the means of 7 measurements.

The results are shown in Table Ⅳ and Fig. 4.

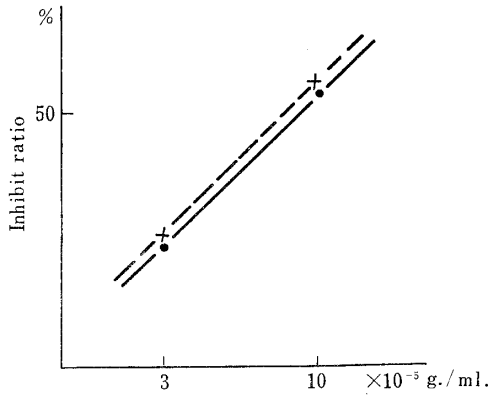
Table Ⅳ. Data for Interaction between Atropine and Parathion against Acetylcholine (5×10^{-8} g./ml.)

Atropine (g./ml.)	Parathion (g./ml.)	Mean inhibitory ratio (%)
2×10^{-9}	—	17.7
2×10^{-9}	—	60.1
—	3×10^{-7}	0
2×10^{-9}	3×10^{-7}	37.9
5×10^{-9}	3×10^{-7}	75.5



parallelism $0.26<F^1_{18}$ 4.41
preparation $14.34>F^1_{18}$ 4.41

Fig. 4. The Effect of Interaction between Atropine and Parathion
The F-test was determined in p=0.05.



parallelism $0.04<F^1_{18}$ 4.41
preparation $0.36<F^1_{18}$ 4.41

Fig. 5. The Effect of Interaction between Pyridine Aldoxime Methiodide and Atropine
The F-test was determined in p=0.05.

From Table Ⅳ, and Fig. 4, it may be estimated that both sites of action of atropine and parathion are respectively independent.

(b) PAM and atropine

The experimental design of this pair consists of two doses 3×10^{-5}, 10^{-4} g./ml. of PAM against 3×10^{-10} g./ml. atropine and the data were made from the means of 7 measurements.

The results are shown in Table Ⅴ, and Fig. 5.

Table V. Data for Interaction between Atropine and Parathion against Acetylcholine (5×10^{-8} g./ml.)

PAM (g./ml.)	Atropine (g./ml.)	Mean inhibitory ratio (%)
3×10^{-5}	—	24.9
1×10^{-4}	—	53.4
—	3×10^{-10}	0
3×10^{-5}	3×10^{-10}	26.2
1×10^{-4}	3×10^{-10}	56.2

From Table V, and Fig. 5, it may be estimated that both sites of action of PAM and atropine are dependent.

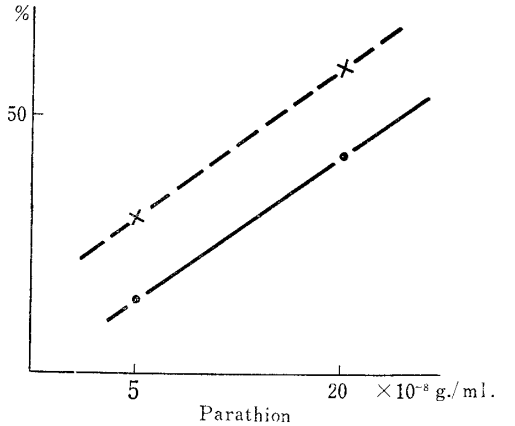
(c) Parathion and PAM

The experimental design of this pair consists of doses 5×10^{-8}, 2×10^{-7} g./ml. of parathion against 3×10^{-6} g./ml. PAM, and the data were made from the means of 6 measurments.

The results are shown in Table VI and Fig. 6.

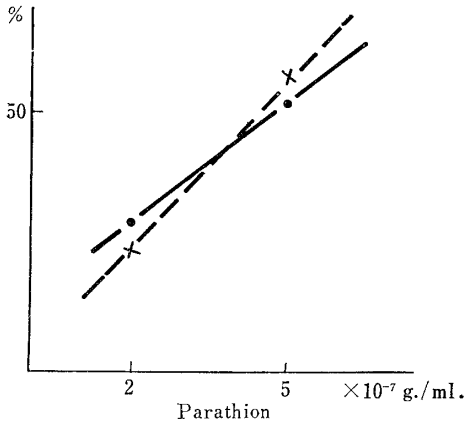
Table VI. Data for Interaction between Parathion and Pyridine Aldoxime Methiodide against Acetylcholine (5×10^{-8} g./ml.)

Parathion (g./ml.)	PAM (g./ml.)	Mean inhibitory ratio (%)
5×10^{-8}	—	13.7
2×10^{-7}	—	42.8
—	3×10^{-6}	0
5×10^{-8}	3×10^{-6}	29.4
2×10^{-7}	3×10^{-6}	59.8



parallelism $0.01<F^1_{15}$ 4.54
preparation $7.98>F^1_{15}$ 4.54

Fig. 6. The Effect of Interaction between Parathion and Pyridine Aldoxime Methiodide
The F-test was determined in p=0.05.



parallelism $7.68>F^1_{15}$ 4.54
preparation $0.73<F^1_{15}$ 4.54

Fig. 7. The Effect of Interaction between Parathion and Isopentyl Acetate
The F-test was determined in p=0.05.

From Table VI and Fig. 6, it may be estimated that both sites of action of parathion and PAM are respectively independent.

(d) Parathion and isopentyl acetate

The experimental design of this pair consists of two doses 2×10^{-7}, 5×10^{-7} g./ml. of parathion against isopentyl acetate 10^{-4} g./ml. and the data were made from the means of 6 measurements.

The results are shown in Table VII and Fig. 7.

Table VII. Data for Interaction between Parathion and Isopentyl Acetate against Acetylcholine (5×10^{-8} g./ml.)

Parathion (g./ml.)	Isopentyl acetate (g./ml.)	Mean inhibitory ratio (%)
2×10^{-7}	—	28.0
5×10^{-7}	—	52.5
—	1×10^{-4}	0
2×10^{-7}	1×10^{-4}	23.4
5×10^{-7}	1×10^{-4}	57.1

From Table VII and Fig. 7, it may be estimated that both sites of action of parathion and isopentyl acetate are dependent.

III. Interaction Effects between Pyridine Aldoxime Methiodide and Isopropyl Diethyl Phosphate or Butyl Diethyl Phosphate

The experimental design consists of two pairs of observation; (a) two doses 3×10^{-5}, 9×10^{-5} g./ml. of PAM against isopropyl diethyl phosphate (IDP : 2×10^{-4} g./ml.), (b) two

Table VIII. Data for Interaction between Pyridine Aldoxime Methiodide and Isopropyl Diethyl Phosphate or Butyl Diethyl Phosphate against Acetylcholine (5×10^{-8} g./ml.)

	PAM (g./ml.)	IDP (g./ml.)	BDP (g./ml.)	Mean inhibitory ratio (%)
(a)	3×10^{-5}	—		17.7
	9×10^{-5}	—		46.8
	—	2×10^{-4}		0
	3×10^{-5}	2×10^{-4}		25.5
	9×10^{-5}	2×10^{-4}		60.7
(b)	2.5×10^{-5}		—	23.8
	1×10^{-4}		—	65.2
	—		5×10^{-5}	0
	2.5×10^{-5}		5×10^{-5}	30.0
	1×10^{-4}		5×10^{-5}	69.4

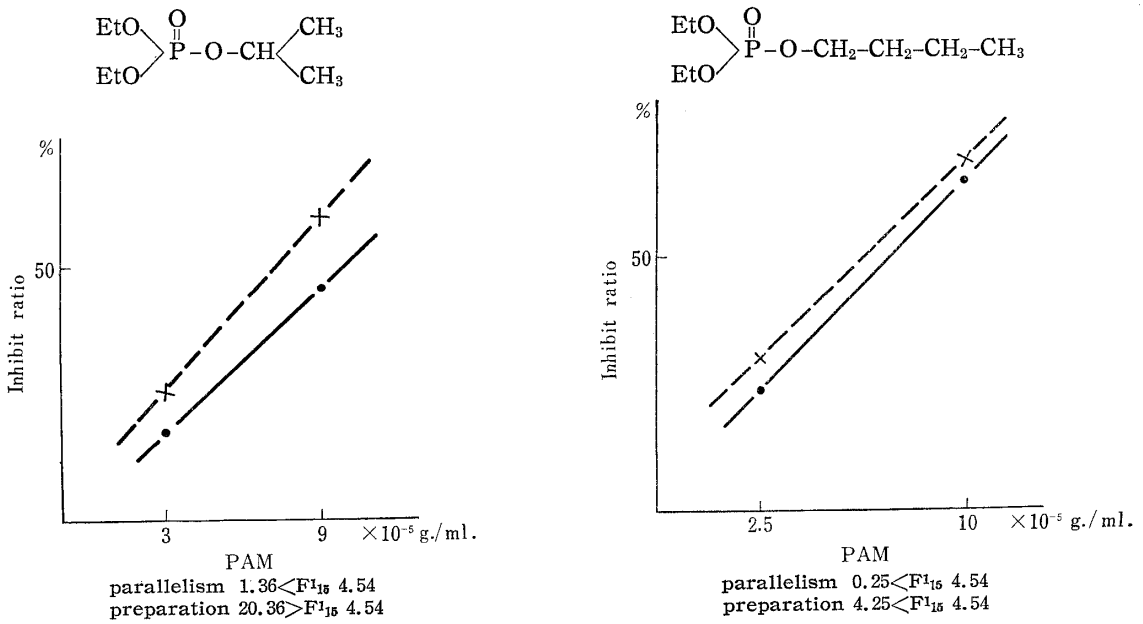


Fig. 8. The Effect of Interaction between Pyridine Aldoxime Methiodide and Isopropyl Diethyl Phosphate (in the left hand), or Butyl Diethyl Phosphate (in the right hand)

doses 2.5×10^{-5}, 10^{-4} g./ml. of PAM against butyl diethyl phosphate (BDP : 5×10^{-5} g./ml.). The results, which are respectively made from the means of 6 measurements, are shown in Table Ⅷ and Fig. 8.

As may readily be seen, from Table Ⅷ and Fig. 8, there are clearly the difference between the results of the two experiments a and b. It may be concluded, therefore, that the influence of IDP molecule is different from that of BDP molecule upon the approach of PAM to the anionic site of ACh surface.

Discussion and Conclusions

In the case of the association of two inhibitors with the same site, it was apparent that all the curve c was overlapped the curve b without shifting to the right hand. Because, the dose of one of two inhibitors (corresponding to an antagonist C) was administered less than the dose of another caused the 50% inhibition. As the natural example in the case of the association of two inhibitors with the same site, the result I was designed with two atropine doses. The results showed that two curves were overlapped. Since this agrees with the fact that the 40.9% as the inhibited effect of atropine is corresponding with the 42.2% as the effects of the half doses for the inhibited effect 65.8% treated with the two times, which were calculated from the data in the previous paper,[8] the evidence adduced to support the stochastical method of the determination can be recognized by the result I.

After surely recognizing such a matter, the results Ⅱ can be discussed with sufficient validity. These results are brought together for ready comparison in Fig. 9. In Fig. 9 it is shown that the interaction between antagonists against the same site on ACh receptor surface is represented with the open circle, and the independent relation against the different site is represented with the cross.

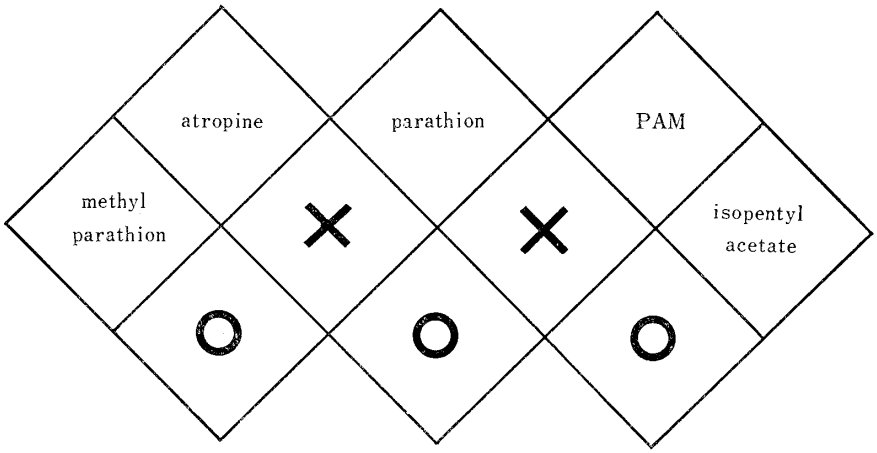


Fig. 9. Diagram illustrating Interaction between Antagonists Approaching to Active Sites on Acetylcholine Receptor Surface
○ : the case of the same active site
× : the case of the different active site

The fact that PAM is not in complete interaction with parathion on the ACh receptor different from the case on the ChE seems to justify the conclusions of PAM obtained in a preceding paper.[1] From Fig. 9, also it is clear that organophosphate group of parathion seems to correspond with ester group of isopentyl acetate, because the action of parathion is independent of PAM and atropine containing quaternary ammonium

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

salt, but is parallel to isopentyl acetate containing ester group only. It has been recognized, of course, that all dosage used in this result II is the competitive inhibitory doses of parathion,[1] PAM,[1] atropine,[8] and isopentyl acetate.[9] According to Pfeiffer,[10] Welsh and Taub,[11] and Barlow,[12] therefore, if a complementary site of the appendage structure of ACh molecule may be assumed in the ACh receptor surface, it may be concluded that organophosphate group is attracted to, what is called, the esteratic site of ACh receptor surface in the inhibitory action of parathion against ACh.

On the other hand, from many suggestions of Barlow,[13] Paton,[14] Schueler,[15] Land and Cavallito,[16] Ormerod,[17] Ohki,[18] and Schild[19] on the feature of ACh receptor surface, it is assumed that the ACh receptor surface has two active sites, anionic site and esteratic site, resembling to the active sites of ChE surface. Assuming that it is true, it will be suggested as the possible mechanism that butyl diethyl phosphate approaches its phosphate group to the esteratic site of ACh receptor surface in the inhibitory action to ACh and yet is to cover the anionic site, where is at the distance of 7 Å with the butyl radical of the phosphate at the same time, so that, even if the other antagonist containing quaternary ammonium salt for instance PAM is added, it will be not possible to approach to anionic site. This suggestion was supported well enough by the results III. As Fig. 8 has already shown, the fact that the interaction between PAM and butyl dietyl phosphate are recognized but PAM and isopropyl diethyl phosphate are independent means that butyl diethyl phosphate can be to hinder the approach of PAM to the anionic site of ACh receptor surface, but isopropyl diethyl phosphate can not do so. Fig. 10 illustrates the possible mechanism of the steric hindrance by phosphate derivatives in the form of scheme.

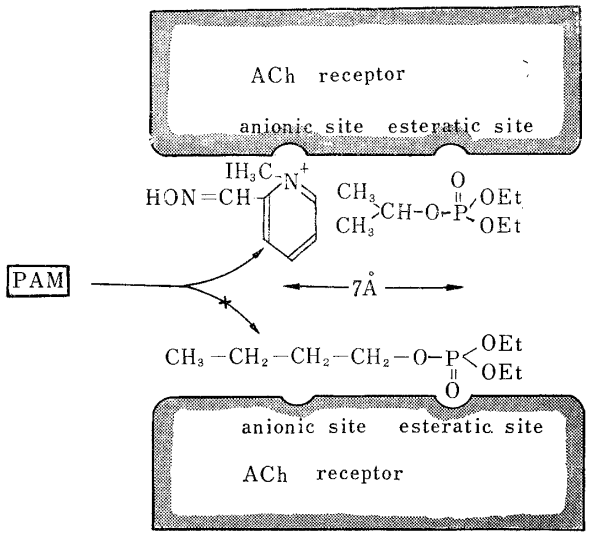


Fig. 10. Scheme to Illustrate a Possible Mechanism of the Steric Hindrance by Butyl Radical of Butyl Diethyl Phosphate against the Approach of Pyridine Aldoxime Methiodide to the Anionic Site of Acetylcholine Receptor Surface

Consequently, these observations were to be regarded as an experimental evidence that there are an anionic site and an esteratic site with the distance of 7 Å on the ACh receptor surface, and yet the site of action of organophosphate group is the esteratic site.

Thanks are given to Mr. Isamu Saikawa and Mr. Hideo Shimasaki for assistance in the experimental work.

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18) K. Ohki: "Saiboh-ryoshi-kagaku" Nankodo, 204 (1957).
19) H.O. Schild: J. Physiol., **153**, 26 (1960).

Summary

In order to determine the site of action of organophosphate group on acetylcholine (ACh) receptor surface, the effect of several antagonists of ACh has been observed with Magnus method using the isolated intestine of mice. These experiments gave following results :

1) A modified method for determining the site of action of antagonist on ACh receptor surface was established stochastically and the validity was evidenced by two trial experiments.

2) According to this justified method, the anticholinergic effect of four pairs consisting of two antagonists were demonstrated and from these results, four pairs were divided into groups acting on the same site and on the different site; the former is two pairs consisting of pyridine aldoxime methiodide (PAM) and atropine, and parathion and isopentyl acetate, the latter is two pairs consisting of atropine and parathion, and parathion and PAM. From this fact, it was concluded that organophosphate group of parathion is attracted to, what is called, the esteratic site of ACh receptor surface in the inhibitory action against ACh.

3) From the fact that the interaction between PAM and butyl diethyl phosphate is recognized but PAM and isopropyl phosphate are independent each other in the inhibitory action against ACh, it was suggested as the possible mechanism that the butyl radical of phosphate can be to hinder the approach of PAM to the anionic site of ACh receptor surface. This is an experimental evidence that there are two active sites with the distance of 7 Å on ACh receptor surface.

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22. Masayasu Kimura*1 and Isamu Saikawa*2 : Molecular Pharmacological Studies on Drug-Receptor Complexes System in Drug Action. IV.*3 Relationship between Anticholinergic and Anticholinesterase Activities of Organophosphoryl Choline Derivatives based on their Chemical Structure.*4

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In an early series of study on organophosphorus compounds, Kimura,[1] one of the authors, found that parathion inhibits acetylcholine (ACh) action on smooth muscle in addition to anticholinesterase (ChE) action, and then with his co-workers he[2] confirmed that ACh molecule is competitively inhibited by parathion molecule at ACh receptor. It has been experimentally concluded by means of pharmacological method that substance

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*2 8-Shimookui, Toyama (才川 勇).
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*4 This study was supported in part by Grant-in-Aid for Fundamental Scientific Research from the Ministry of Education, and presented at the 81th annual meeting of the Pharmaceutical Society of Japan (July 1961, in Sapporo).
1) M. Kimura : This Bulletin, **11**, 44 (1963).
2) M. Kimura, T. Igarashi, S. Iwashita : *Ibid.*, **11**, 51 (1963).