[Chem. Pharm. Bull. 12 (2) 145 ~ 150] UDC 612.398.145

20. Morio Ikehara, Eiko Ohtsuka, and Yoshihiro Kodama : Studies of Nucleosides and Nucleotides. XXII.*1 Phosphorylation of Adenosine using Borate Complex as Protecting Group for 2′- and 3′-Hydroxyl Groups.

(*Faculty of Pharmaceutical Sciences, School of Medicine, Hokkaido University**2)

As discussed in the previous paper of this series,[1] it became necessary to establish the method for the direct phosphorylation of primary hydroxyl group of nucleosides. As an approach we have investigated with lupetidyl phosphorodichloridate,[1] which was bearing a bulky protecting group and was expected to phosphorylate exclusively the most freely accessible primary hydroxyl group. However, the experiments resulted in a limited success.

In this paper another approach with borate complex as a protecting group on vicinal hydroxyl group of naturally occurring nucleoside is reported. It is well known[2,3] that boric acid forms a complex ester as shown below. The complex is fairy stable in such an appropriate condition as was substantiated in the protection of carbohydrate hydroxyl groups during acylation reaction.[4~6]

Considering these facts, it is worthwhile to investigate the possibility of protection with borate complex during phosphorylation of nucleoside. In order to compare the results of the experiments with those in the absence of boric acid, three phosphorylating agents, morpholino phosphorodichloridate,[7] P^1-diphenyl P^2-morpholino pyrophosphorochloridate[8] and 2,6-lupetidyl phosphorodichloridate[1] were used.

DMF*3 was used as the solvent of the reaction, because it was the most favorable solvent to solubilize most of the nucleosides.

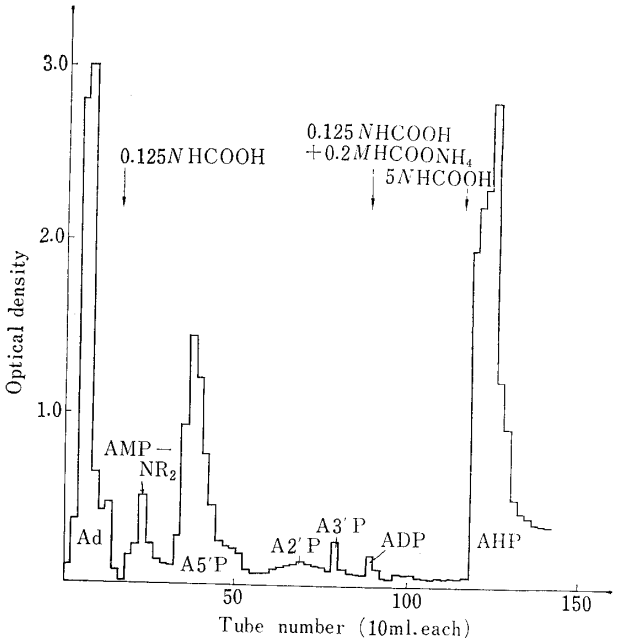


Fig. 1. Chromatographical Pattern of Phosphorylation of Adenosine with Morpholino Phosphorodichloridate in the Presence of Boric Acid

*1 Part XXI. M. Ikehara, E. Ohtsuka : This Bulletin, **11**, 1358 (1963).
*2 Kita 12-jo, Nishi 5-chome, Sapporo (池原森男, 大塚栄子, 児玉喜博).
*3 Abbreviations used are as follows : DMF, N,N-dimethylformamide; MP, monophosphate; DP, diphosphate; HP, higher phosphate.

1) M. Ikehara, E. Ohtsuka, Y. Kodama : This Bulletin, **11**, 1456 (1963).
2) J. Böeseken : "Advances in Carbohydrate Chemistry," **4**, 189 (1949).
3) J. X. Khym, W. E. Cohn : Biochim. Biophys. Acta, **15**, 139 (1954).
4) L. v. Vargha : Ber., **66**, 704 (1934).
5) M. Akagi, S. Tejima : This Bulletin, in preparation.
6) The use of borate complex for the acylation of nucleoside was briefly reported (Abstracts of Papers presented in 12th Annual Meeting of Chemical Society of Japan, p. 171).
7) M. Ikehara, E. Ohtsuka : This Bulletin, **11**, 435 (1963).
8) *Idem* : *Ibid.*, **11**, 961 (1963).

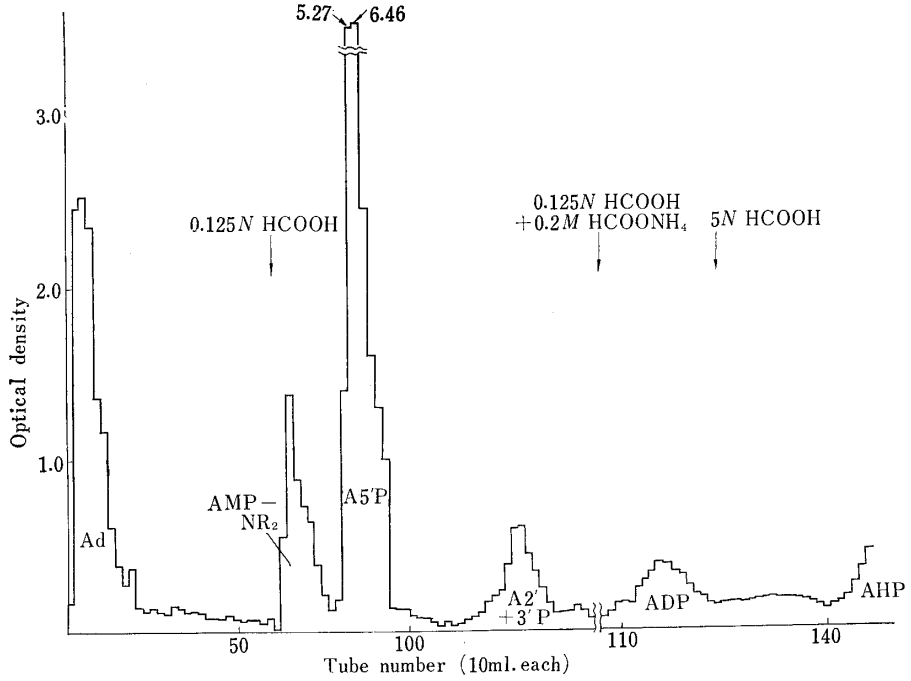


Fig. 2. Chromatographical Pattern of Phosphorylation of Adenosine with P^1-Diphenyl P^2-Morpholino Phosphorodichloridate in the Presence of Boric Acid

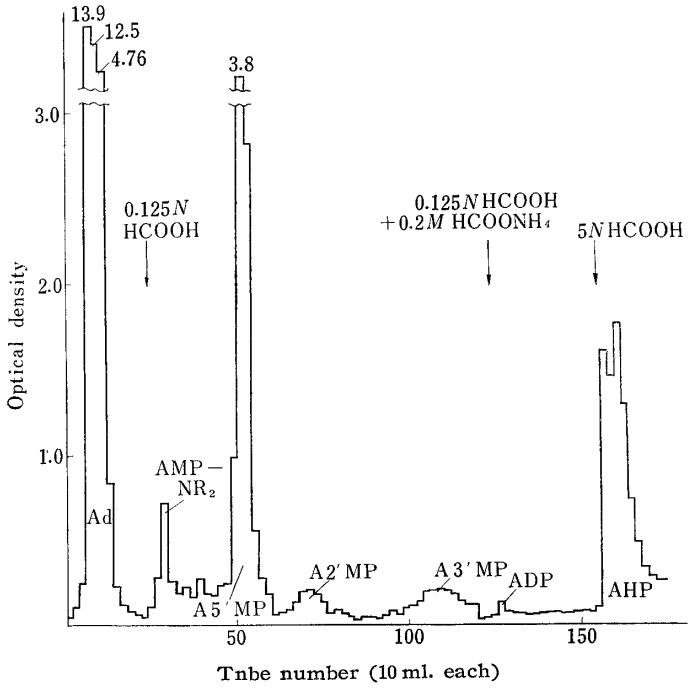


Fig. 3. Chromatographical Pattern of Phosphorylation of Adenosine with Lupetidyl Phosphorodichloridate in the Presence of Boric Acid

Adenosine was dissolved in DMF with gentle warming and then cooled. Equimolar (or slight excess) amount of boric acid to adenosine was added into the cooled solution. A clear solution was obtained. Into this solution two moles of phosphorylating agent were added together with acid acceptor amine. The reaction was carried out under the condition indicated in Table I. The extent of the reaction proceeded was estimated by paper electrophoresis, by which 40~60% of phosphorylation was observed. After the reaction was over, excess water was added and the whole solution was heated at 100° for 45 minutes in order to hydrolyze morpholidate and unreacted reagent. In the experiment using P^1-diphenyl P^2-morpholino pyrophosphorochloridate, diphenyl hydrogen phosphate was extracted at this pH with ether. The solution was evaporated *in vacuo* to a small bulk and co-distilled with a large excess of methanol[9] to remove boric acid as methyl ester. The pH of the solution was adjusted to 8.5 and amines were extracted with ether. A part of the solution was applied to the ion-exchanger chromatography on Dowex I formate column. Elution

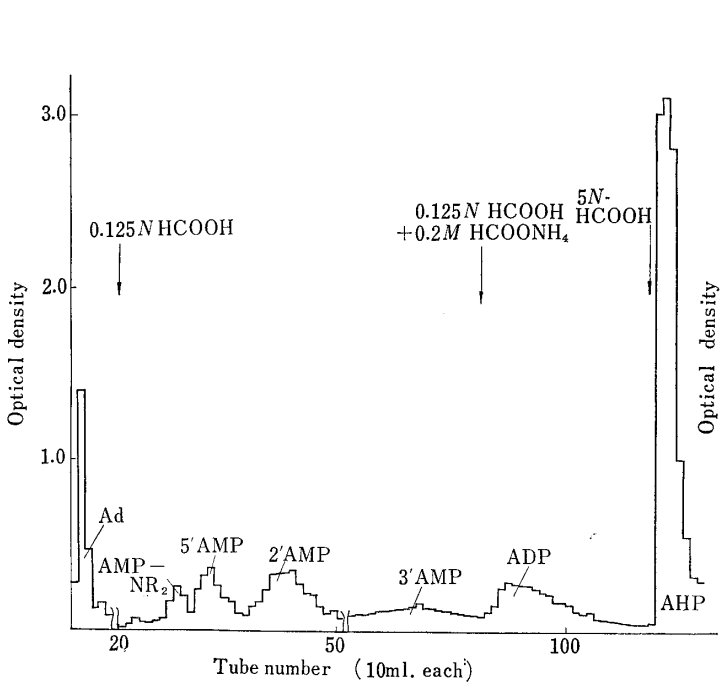


Fig. 4. Chromatographical Pattern of Phosphorylation of Adenosine with Morpholino Phosphorodichloridate

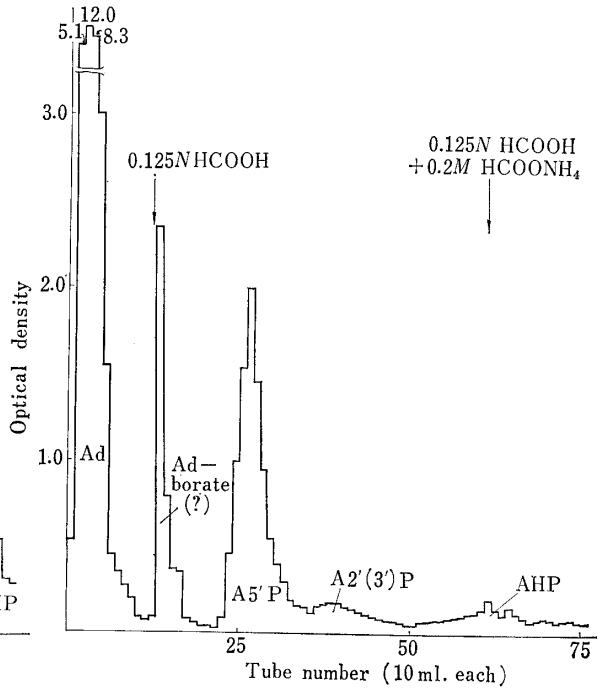
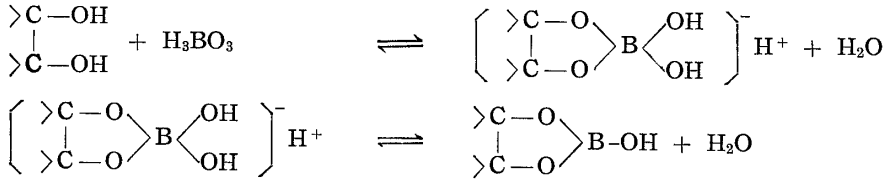


Fig. 5. Chromatographical Pattern of Phosphorylation of Adenosine with Lupetidyl Phosphorodichloridate without Mthanol Treatment

with formic acid and ammonium formate solution gave adenosine, adenosine 5′-, 2′-, 3′-monophosphate, adenosine diphosphates and higher phosphates in this order. The percentage yield of the products was summarized in Table I, together with those obtained in the previous experiments[1,10].



Structure of Boric Acid Complex and Neutral Ester

Fractions obtained in chromatography were neutralized with ammonia, evaporated to a small bulk and tested for their homogeneity by paper chromatography and paper electrophoresis. Rf and R_{AMP} values were summarized in Table II.

Above results gave the following facts: i) The phosphorylation occurred in the order of P^1-diphenyl P^2-morpholino pyrophosphorochloridate>morpholino phosphorodichloridate>lupetidyl phosphorodichloridate. ii) The ratio of the amount of 5′-MP to the sum amount of 2′- and 3′-MP was shown in the decreasing order as same as in i). iii) The ratio in the presence of boric acid was always larger than that in the absence of boric acid. iv) The amount of higher phosphates was in the order of morpholino phosphorodichloridate>lupetidyl phosphorodichloridate>P^1-diphenyl P^2-morpholino pyro-

9) When this treatment was omitted, the elution pattern of the ion-exchanger chromatography revealed as having a complex nature (as shown in Fig. 5). This phenomena may be explained by the presence of borate complex, which increased the holding tendency of the products to the ion-exchanging resin.

10) M. Ikehara, E. Ohtsuka: This Bulletin, **11**, 1353 (1963).

Table I. Results of Phosphorylation of Adenosine with Morpholino Phosphorodichloridate (Reagent I), P^1-Diphenyl P^2-Morpholino Pyrophosphorochloridate (Reagent II) and Lupetidyl Phosphorodichloridate (Reagent III)

Exptl. No.	Rg.	H_3BO_3	Temp. (°C)	Time (hr.)	Yield of products (%)							Ratio (5′-MP/2′- + 3′-MP)	Reference
					Ad	AMP-NR_2	5′-MP	2′-MP	3′-MP	DP	HP		
1	II	+	RT	48	34.5	4.96	27.6	5.01		—	—	5.5	
2	II	+	30	48	13.5	4.57	19.5	4.22		3.7	25.0	4.6	
3	I	+	RT	48	16.2	6.70	1.37	—	—	—	—		a)
4	I	+	30	48	19.5	2.65	11.5	1.93	1.19	1.34	34.3	4.0	
5	III	+	75	2.5	29.0	4.8	11.2	1.54	1.12	—	—		a)
6	III	+	75	2.5	49.1	3.95	14.8	2.25	3.14	0.63	16.4	2.7	
7	III	—	75	2.5			15.0	3.0	5.1		—	1.9	1)
8	II	—	20	48	7.3	1.8	3.5	1.9	4.6	17.4		0.54	10)
9	I	—	20	48	4.1	1.3	3.5	4.5	3.5	5.1	18.3	0.58	

a) MeOH treatment was omitted.

Table II. R_{AMP} and Rf Values of the Individual Phosphates and Adenosine

Substance	R_{AMP}	Solvent			
		A[a]	B[b]	C[b]	D[b]
Adenosine	0.22	0.38	0.20	0.49	0.61
AMP-NR_2	0.72			0.37[c]	
A-5′-MP	1.00	0.33		0.21	0.38
A-2′(3′)-MP	1.00	0.30		0.31	0.45
ADP	1.31				
Pi	1.63	0.49		0.17	0.33

a) Performed by descending technique.
b) Performed by ascending technique.
c) NR_2 indicated morpholine.

phosphorochloridate. v) The reaction proceeded more extensively in the presence of boric acid than in its absence.

The order of the phosphorylating power was coincided with those observed previously in the reaction of 2′,3′-protected nucleoside.[7~12] P^1-Diphenyl P^2-morpholino pyrophosphorochloridate exerted much higher phosphorylating activity than morpholino phosphorodichloridate, although in the DMF solution the former reagent had been inhibited drastically.[9]

As judged from the ratio of 5′-MP *vs.* 2′- + 3′-MP, boric acid was useful as the protecting group of vicinal hydroxyl groups. Formation of the complex was limited by the equilibrium and its stability depended on the nature of the environment. A basic reaction media, at least in the initial stage of the reaction investigated, may dissociate the complex partly and the phosphorylation of 2′- and 3′-hydroxyl has not exclusively been avoided. Furthermore, a part of the phosphorylating agent may be destructed by the attack of the borate. Despite of these drawbacks boric acid complex increased the ratio of 5′-MP *vs.* 2′- and 3′-MP from 0.54~1.9 to 2.7~5.5, which appeared in Table I, experiment 1~6.

The formation of the higher phosphates may be explained by the bifunctional nature of the phosphorodichloridate type reagent.

11) M. Ikehara, E. Ohtsuka : This Bulletin, **11**, 1358 (1963).
12) M. Ikehara, E. Ohtsuka, S. Kitagawa, Y. Tonomura : Biochim. Biophys. Acta, in press.

Although the reason why always higher reaction extent was observed in the presence of borate complex than in its absence was not clear yet, the amount of higher phosphates seems to indicate enhancement of phosphorylating power of the reagent used. Experiments for elucidation of this point and the use of other phosphorylating reagents are now under way.

Experimental

Paper Chromatography——All chromatographies were performed on Toyo Filter Paper No. 51A. Solvent A, iso-PrOH-1% $(NH_4)_2SO_4$=2:1; solvent B, BuOH-H_2O=86:14; solvent C, iso-PrOH-NH_4OH-H_2O=7:1:2; solvent D, PrOH-NH_4OH-H_2O=55:35:10.

Paper Electrophoresis——All electrophoreses were performed on Toyo Filter Paper No. 51A. Condition, 0.05*M* triethylammonium bicarbonate, pH 7.5, 20 volt/cm., 1 hr.

General Procedure——Well dried (heated at 60° over P_2O_5 for 3 hr. in a desiccator under 3 mm.Hg) adenosine (93 mg., 0.3 mmole) was dissolved in 4 ml. of DMF by a slight warming and after cooling to room temperature 0.6 mmole of phosphorylating agent dissolved in 2 ml. of DMF (or dioxane) and 0.6 mmole of triethylamine (or 2,6-lutidine) were added. After the reaction in the condition indicated in Table I, an aliquot was examined by paper electrophoresis and paper chromatography. Reaction extent was estimated photometrically from the UV absorption of the H_2O-extract of the appropriate spot. H_2O (50 ml.) was added into reaction mixture (pH became 2.0~2.4) and the whole solution was heated at 100° for 45 min. on a water bath. After concentration to a small bulk under reduced pressure at 25~30°, MeOH (100 ml.) was added and evaporated *in vacuo*. MeOH (100 ml.) was added again into the residue and evaporated. The residual syrup was taken up in 2 ml. of H_2O and pH of the solution was adjusted to 8.5 with NH_4OH. Amines were extracted with Et_2O (10 ml.×3) and the H_2O-layer was diluted upto 100 ml. in a volumetric flask. One tenth of the solution was applied to the ion-exchange chromatography (Dowex-1×8, formate, 200~400 mesh, 0.8×21 cm.). Column was eluted with 0.125*M* HCOOH, 0.125*M* HCOOH and 0.2*M* $HCOONH_4$, and 5*N* HCOOH by the stepwise elution technique (each fraction was 10 ml.). Fractions containing appropriate products were collected, neutralized with NH_4OH and concentrated to a small bulk in a rotary evaporator at 25~30°. An aliquot was examined by paper chromatography and electrophoresis in the several solvent systems (Rf and R_{AMP} values were listed in Table II) and compared with authentic samples by co-chromatography. Yield of the product was calcuated from the optical density units of the fractions obtained in chromatography. Each fractions were tested for their homogeneity by the above chromatographical test. The concentrated solutions were tested also for the absence of H_3BO_3 by flame test.[13]

Phosphorylation with Morpholino Phosphorodichloridate and 2,6-Lupetidyl Phosphorodichloridate——In these experiments DMF was used as the solvent. Morpholino phosphorodichloridate (122 mg., 0.6 mmole) or 2,6-lupetidyl phosphorodichloridate (124 mg., 0.6 mmole) and 61 mg. (0.6 mmole) of Et_3N were used. Reaction extent estimated by paper electrophoresis was 51 and 46% respectively. Other results were listed in Table I.

Phosphorylation with P^1-Diphenyl P^2-Morpholino Pyrophosphorochloridate——Reagent was freshly prepared from morpholino phosphorodichloridate (1.2 mmoles, 245 mg.), diphenyl hydrogen phosphate (1.2 mmoles, 300mg.) and 2,6-lutidine (2.4 mmoles, 272 μl.) in 2 ml. of dry dioxane. This was directly added to adenosine (0.6 mmole, 161 mg.) and H_3BO_3 (0.6 mmole, 37 mg.). Reaction extent after 48 hr. was 60%. Results were listed in Table I.

Phosphorylation of Adenosine with Morpholino Phosphorodichloridate without Addition of Boric Acid——H_3BO_3 was omitted from the above reaction mixture using morpholino phosphorodichloridate as phosphorylating agent. Reaction extent was 40%. MeOH evaporation was not performed. Results were listed in Table I.

Enzymatic Characterization of Adenosine 5′-MP——Fractions corresponding to 5′-AMP, which was examined by the pattern of ion-exchanger chromatography, paper chromatography and paper electrophoresis, were neutralized with NH_3 and evaporated *in vacuo* to a syrup. The content of 5′-AMP was estimated photometrically from the optical density of the solution. 5′-AMP diammonium salt (ca. 10 μ moles) dissolved in 1 ml. of H_2O was taken and incubated with 0.2 ml. of 0.2*M* tris buffer (pH 6.0), 0.1 ml. of 0.3*M* $MgCl_2$ and 0.1 ml. of crude snake venom[14] (A_{280}=1.3, ca. 100 μg.) at 37° for 4.5 hr. After quenching of the reaction by heating for 10 min. at 100° in a water bath, an aliquot was examined by paper electrophoresis and paper chromatography. Spot corresponding to 5′-AMP was diminished and those of adenosine and inorganic phosphate was revealed by UV irradiation and molibdate spray[15] (Rf values were listed in Table II).

13) F.P. Tradwell, W.T. Hall: "Analytical Chemistry," Vol. **I**, 376 (1937).
14) Y. Mizuno, M. Ikehara, T. Ueda, A. Nomura, E. Ohtsuka, F. Ishikawa: This Bulletin, **9**, 338 (1961).
15) C.S. Hanes, F.A. Isherwood: Nature, **164**, 1107 (1949).

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.

(Received October 21, 1963)

[Chem. Pharm. Bull. 12 (2) 150 ~ 159]

UDC 615.711.7-015

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

(*Faculty of Pharmaceutical Sciences, University of Toyama**1)

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.

---

*1 5-Okuda, Toyama (木村正康).

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

1) Part II. M. Kimura, T. Igarashi, S. Iwashita : This Bulletin, **11**, 51 (1963).
2) I.B. Wilson, F. Bergman : J. Biol. Chem., **185**, 479 (1950).
3) I.B. Wilson : *Ibid.*, **199**, 113 (1952).
4) D.R. Davies, A.C. Green : Biochem. J., **63**, 529 (1956).