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1. Masayasu Kimura: Molecular Pharmacological Studies on Drug-Receptor Complexes System in Drug Action. V.*1 The Comparison of the Active Sites of Acetylcholine Receptor and Cholinesterase Surface.*2

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It is well known that both Acetylcholine (ACh) and Cholinesterase (ChE) at the neuromuscular junction play an important role in the transmission of the peripheral nervous systems. For a long time many studies have brought forward considerable evidences to support the hypothesis that the function of ACh in the neurohumoral transmission is discharged by the complex formation between ACh and its receptor on the cell surface of the effector organ. Nevertheless, the reports which deal with the exact relationship between ACh receptor and ChE are extremely scanty. In the pharmacological field, especially in the study of the interaction between a drug and its receptor, it must be of the first importance to decide whether the enzyme could be the receptor substance itself or not.

Zupancic[1] described that the receptive substance for ACh was identical with ChE. According to the review of Nachmansohn and Wilson,[2] the active site of ACh receptor is different from that of ChE.

The author have already shown that only the anionic site of ACh receptor is similar to that of ChE,[3] while it was suggested that there may be a distinct difference between ACh receptor and ChE themselves.[4,5]

The purpose of this study is to determine whether ACh receptor is identical with ChE or not at the pharmacological approach. Furthermore using a valuable tool of organophosphoryl choline, which had been found by the authors,[5] it is another object of this study to investigate the difference between ACh receptor and ChE.

Methods and Materials

1) Pharmacological Procedure for Drug-Receptor Reaction——Magnus's method was adapted using the rectus abdominis of frog (*R. nigromaculate*) weighing 25~35 g. A muscle bath was filled with 5 ml.

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*1 Part Ⅳ: This Bulletin, **12**, 159 (1964).

*2 This was published at the 82nd Annual Meeting of Pharmaceutical Society of Japan in Shizuoka (Nov. 2, 1962).

*3 Gofuku, Toyama (木村正康).

1) A. O. Zupancic: Acta. Physiol. Scand., **29**, 63 (1953).

2) D. Nachmansohn, I. B. Wilson: "Currents in Biochemical Research" by chief editor D. E. Green, 628 (1956).

3) M. Kimura: This Bulletin, **7**, 837 (1959).

4) *Idem*: *Ibid.*, **11**, 44 (1963).

5) M. Kimura, I. Saikawa: *Ibid.*, **12**, 159 (1964).

of Ringer solution at room temperature and it was constantly agitated by the bubbling air through the solution. The muscle was always rested for at least 10 min., or for 20 min. in the presence of antagonists. The response of contractions was recorded on kymographion and was expressed in percentage of the maximum response caused by a excessive dose of agonist. Antagonist was applied 3~5 min. before the application of an agonist.

2) **Biochemical Procedure for Enzyme Action**——Warburg's manometric method[6] was used for measuring a inhibition of ChE by organophosphates in Ringer solution. The Enzyme preparation was powder of dried pseudo ChE which was prepared by lyophilization from the blood serum of rabbit weighing about 2 kg. as follows. After the blood was collected through the auricularis artery, it was kept in an ice box until it coagulated in a glass vessel. The cold blood was centrifuged at 3000 r.p.m. for 5~10 min., and the serum was lyophilized. The product was dissolved in Ringer solution, and was recentrifuged at 2000 r.p.m. for 5~10 min. followed by lyophilization. The resultant powder was used as the ChE preparation. The final concentration using in the experiment was 10^{-2} g./ml. of dried blood serum and 1.5×10^{-2} g./ml. of dried erythrocyte for the hydrolysis against $1.1\times10^{-3}M$ of ACh at 37°.

3) **The Isotonic Buffer Solution used in Biological Experiment for the Influence of pH, and the Procedure for Determining of the Apparent pKa of the Active Site**——In the case of muscle preparation, the isotonic modified Ringer solution was made by adding the mixture solution of NaCl 6.5 g./L., KCl 0.2 g./L., and $CaCl_2$ 0.2 g./L. to McIlvaine's buffer solution. In the case of enzyme, the isotonic buffer solution was preparated by the same procedure as above from Kolthoff, Sörensen and Palitzsch buffer solution.

The procedure for determining the apparent dissociation constant pKa of active site in ACh receptor was the same as in the previous paper.[3]

4) **Synthesis of Compounds used as the Biological Agents**——Following compounds[5] were used :

ATMA $CH_3CH_2CH_2CH_2CH_2N^+(CH_3)_3Br^-$

NP214 $\left(\bigcirc\!\!\rangle O\right)_2P(O)OCH_2CH_2N^+(CH_3)_3I^-$

NP224 $\left(\bigcirc\!\!\rangle O\right)_2P(S)OCH_2CH_2N^+(CH_3)_3I^-$

NP244 $\left(\bigcirc\!\!\rangle O\right)_2P(S)SCH_2CH_2N^+(CH_3)_3I^-$

Experimental Results

I. Dose-Response Curve of ATMA——This experiment was conducted to prove that ATMA is possible to substitute for ACh in the interaction between ACh and its receptor on the rectus abdominis muscle of frog. Doses of ATMA were divied into three levels 4.76×10^{-6}, 9.52×10^{-6}, and $1.90\times10^{-5}M$ and then $4.76\times10^{-4}M$ was used for the maximum contraction. Six preparations were used for each dose.

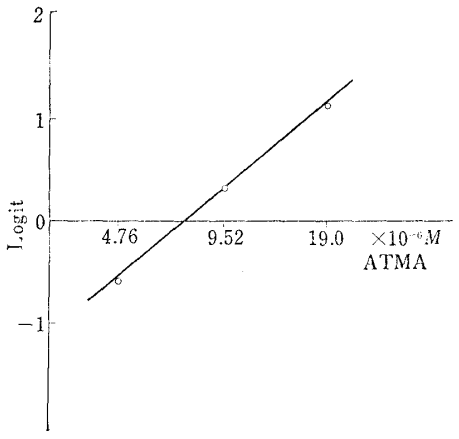


Fig. 1. The Logistic Regression Line of Dose-Response Curve of ATMA on the Rectus Abdominis Muscle of Frog
The slope of curve was estimated as 0.913.

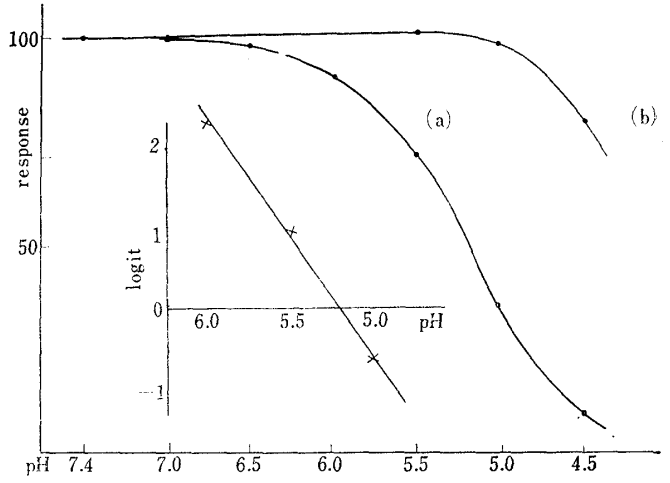


Fig. 2. Dose-Inhibitory Response Curve by the Hydrogen Ion against a Certain Dose of ATMA (a curve : $1.43\times10^{-5}M$, b curve : $4.76\times10^{-4}M$) and its Logistic Regression Line

6) R. Ammon : Pflüger's Arch. Ges. Physiol., **233**, 486 (1933).

The date were analyzed according to the method established in the previous paper.[7] Fig. 1 shows the logistic regression line of dose-response curve of ATMA. In view of the analysis that the slope of ATMA curve does not deviate significantly from the theoretical value of 1, it may be concluded that one molecule of ATMA combines with an ACh receptor in the process of the contractile response like the case of ACh.[7]

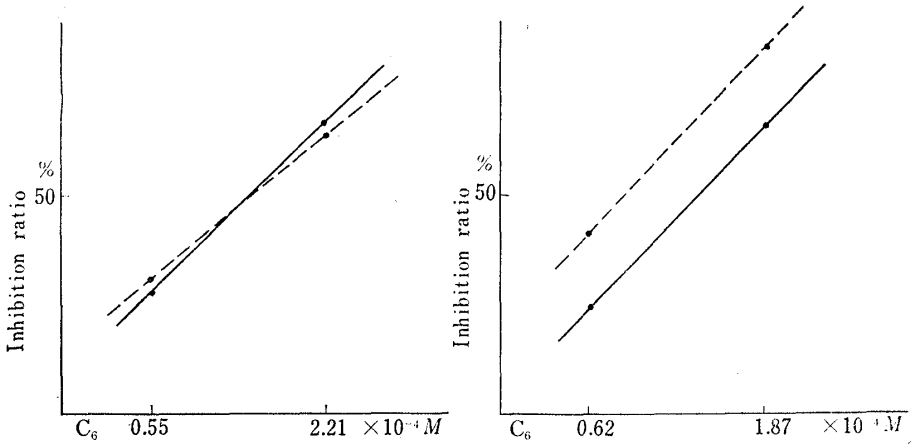
II. Dose-Inhibition Curve by the Hydrogen Ion against ATMA——In this experiment, the inhibitory action of H^+ against the formation of the ATMA-ACh receptor complex was investigated. The results on dose-inhibition curve of the H^+ against 1.43×10^{-5} and $4.76\times10^{-4}M$ of ATMA were shown in Fig. 2. From the result, it was shown that slope of curve of H^+ does not deviate significantly from the theoretical value of 1. This means that a hydrogen ion competes with one molecule of ATMA at ACh receptor.

III. The Site of Action of H^+ on ACh Receptor——The object of this experiment is whether the site of action of H^+ is only the anionic site of ACh receptor. According to the method established in the previous paper,[8] the difference of synergistic effect against ACh between two pairs, that is, (a) H^+ and hexamethonium bromide which clearly acts on the anionic site, and (b) H^+ and triisopropylthionophosphate which was recognized to act on the esteratic site, was observed.

(a) In the case of H^+ and hexamethonium ion : The experimental design of this pair consists of two doses 5.52×10^{-5} and $2.22\times10^{-4}M$ of hexamethonium bromide against $1.1\times10^{-5}M$ of ACh with pH 5.5 and 7.0, and the data were the means of 6 measurements. The results are shown in Fig. 3-a, from which it may be sure that the difference of the sites of action of H^+ and hexamethonium ion is not statistically significant.

(b) In the case of H^+ and triisopropylthionophosphate : The experimental design of this pair consists of two doses 6.24×10^{-5} and $1.87\times10^{-4}M$ of triisopropylthionophosphate against $1.1\times10^{-5}M$ of ACh with pH 5.5 and 7.0 and the data were the means of 6 measurements. The results are shown in Fig. 3-b, from which it may be estimated that both sites of action of H^+ and phosphate group are respectively independent. From the above results, it may be concluded that H^+ affects the anionic site and does not the esteratic site of ACh receptor at all.

(a) H^+-Hexamethonium bromide (b) H^+-Triisopropylthionophosphate



Parallelism $1.12<F_{15}^{1}$ 4.54 Parallelism $0.063<F_{15}^{1}$ 4.54
Preparation $0.008<F_{15}^{1}$ 4.54 Preparation $23.55>F_{15}^{1}$ 4.54

Fig. 3. The Two Graphs illustrate the Difference between (a) Hydrogen Ion and Hexamethonium Bromide and (b) Hydrogen Ion and Triisopropylthionophosphate by Means of the Relation of Two Dose-Inhibitory Response Curves (——•—— pH 7.0, ——•—— pH 5.5)

IV. The Apparent pKa Value of the Anionic Site of ACh Receptor——The two dose-inhibition curves in the range of pH 5.2 and pH 5.6 with the same muscle against 9.52×10^{-6} and $2.86\times10^{-5}M$ of ATMA were obtained by using 9 muscles. These results were shown in Fig. 4, from which it may be estimated that the apparent pKa of the anionic site of ACh receptor is about 5.92.

V. The Association Constant K between the Esteratic Site of ACh Receptor and Triisopropylphosphate——The two dose-inhibition curves of triisopropylphosphate with the same muscle against ACh

7) K. Takagi, M. Kimura : This Bulletin, **6**, 449 (1956); K. Takagi, *et al.* : Yakugaku Zasshi, **76**, 1191 (1956).
8) M. Kimura : This Bulletin, **12**, 150 (1964).

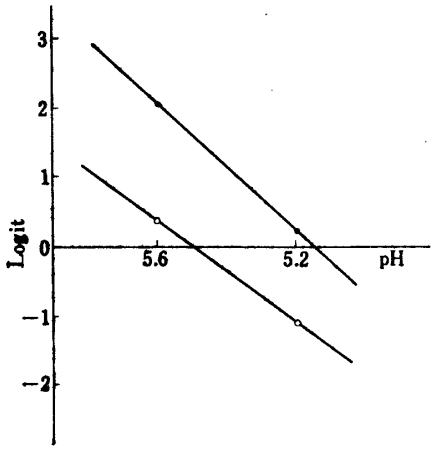


Fig. 4. Dose-Inhibition Curves of Hydrogen Ion against ATMA (upper line: $2.86\times10^{-5}M$, lower line: $9.52\times10^{-6}M$) on ACh receptor

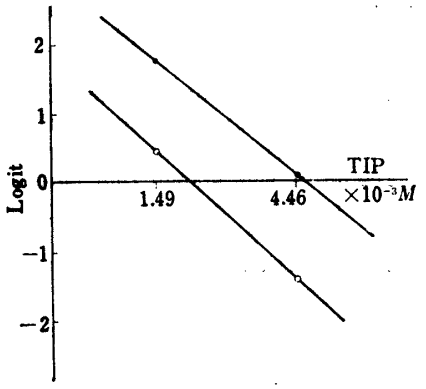


Fig. 5. Dose-Inhibition Curves of TIP against ACh (upper line : $1.38\times10^{-7}M$; lower line : $5.5\times10^{-8}M$) on the Rectus Abdominis Muscle of Frog

(5.5×10^{-8} and $1.38\times10^{-7}M$) were obtained using 10 muscles. The results were shown in Fig. 5, from which it may be estimated that the apparent pK between the esteratic site of ACh receptor and phosphate group is about 3.26.

VI. The Association Constant K between Esteratic Site of ChE and Triisopropylphosphate——In order to compare the esteratic site of ACh receptor with that of ChE, the association constant between ChE and triisopropylphosphate was obtained by two experimental designs of estimation using Warburg's manometric method.

(A) The two dose-inhibition curves consisted of 4.46×10^{-3} and $7.14\times10^{-2}M$ triisopropylphosphate against ACh (1.1×10^{-2} and $4.4\times10^{-3}M$) containing the erythrocytes 1.5×10^{-2} g./ml. were obtained with 7 repeats. These results were shown in Fig. 6, from which it may be estimated that the pK between the esteratic site of ChE and phosphate group is about 2.08.

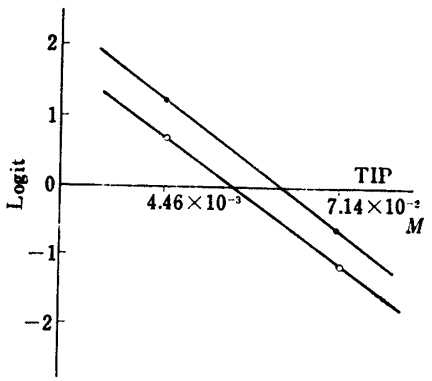


Fig. 6. Dose-Inhibition Curves of TIP against ACh (upper line: $4.4\times10^{-3}M$; lower line : $1.1\times10^{-3}M$) on the ChE

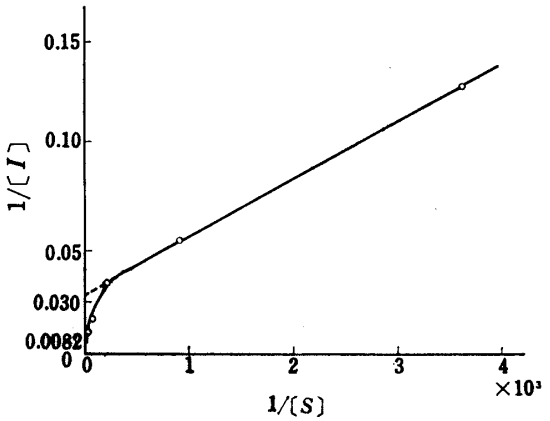


Fig. 7. A Curve between TIP ($1/S$) and its Inhibitory Response ($1/I$) on ChE

Table I. Data of Inhibitory Response TIP against ACh Hydrolysis Effect of ChE

TIP (M)	1/TIP (M)	I (%)	$1/I$ (%)
2.78×10^{-4}	3.60×10^{3}	7.9	1.27×10^{-1}
1.12×10^{-3}	8.93×10^{2}	18.7	5.35×10^{-2}
4.46×10^{-3}	2.24×10^{2}	27.9	3.58×10^{-2}
1.79×10^{-2}	5.59×10	53.6	1.87×10^{-2}
7.14×10^{-2}	1.40×10	77.5	1.29×10^{-2}

(B) By the ordinary design in enzymatic chemistry, a dose inhibition curve in the range of doses from 2.78×10^{-4} to $7.14\times10^{-2}M$ of triisopropylphosphate against ACh $1.1\times10^{-3}M$ containing the erythrocyte 1.5×10^{-2} g./ml. were obtained with 7 repeats. These results were shown in Table I and Fig. 7. Now, in the equation $\frac{1}{I}=\frac{K}{I_{max}}\cdot\frac{1}{TIP}+\frac{1}{I_{max}}$, $1/I=0.03$ is obtained graphically when $1/TIP=0$ from Fig. 7. Therefore, according to data in Table I, K can be calculated as 4.63×10^{-3}. From the results, it may be estimated that the pK between the esteratic site of ChE and phosphate group is about 2.33.

These two pK values obtained from the two different designs of estimation is approximate exceedingly.

VII. The Correlation between Anti-ACh and Anti-ChE Activity of Organophosphorylcholine Derivatives——Anticholinergic activity is shown by 50% inhibitory molar dose (ID_{50}) against ACh $1.1\times10^{-5}M$ in the presence of neostigmine methylsulfate $3\times10^{-6}M$ using 4 muscles. Anticholinesterase activity is represented by ID_{50} against ACh $1.1\times10^{-3}M$ with the erythrocyte preparation (1.5×10^{-2} g./ml.) as true ChE and with serum preparation (1×10^{-2} g./ml.) as pseudo ChE from 3 repeats of measurement with photoelectric colorimetric method. These results were shown in Table II, from which it was indicated that anti-ACh activity increased in the order of $-O-\overset{\overset{O}{\|}}{P}<$, $-O-\overset{\overset{S}{\|}}{P}<$, and $-S-\overset{\overset{S}{\|}}{P}<$, while anti-ChE activity was not parallel with the former in the case of both true and pseudo ChE, with the exception that $-O-\overset{\overset{O}{\|}}{P}<$ and $-O-\overset{\overset{S}{\|}}{P}<$ are approximately in the same order.

Table II. Anti-ACh and Anti-ChE Activity (ID_{50}) of Organophosphorylcholine Derivatives

Derivatives	Anti-ACh	Anti-True ChE	Anti-Pseudo ChE
NP 214 $\left(-O-\overset{\overset{O}{\|}}{P}-O\right)$	4.59	7.03	7.92
NP 224 $\left(-O-\overset{\overset{S}{\|}}{P}-S\right)$	5.91	7.21	7.99
NP 244 $\left(-S-\overset{\overset{S}{\|}}{P}-S\right)$	6.12	5.45	6.90

It is quite possible, therefore, there is a distinct difference between the esteratic site of ACh receptor and ChE surface.

Discussion and Conclusions

In regard to the effect of pH on ACh-receptor complex, since Kimura[3,9] reported on the small intestines of mouse, several workers have published on the illeum of guinea pig,[10] or rabbit,[11] and the uterus of rat.[12] Comparing these results, there is a wide difference of the competitively inhibiting range of hydrogen ion against ACh in various tissues. In this paper, it was shown that the rectus abdominis muscle of frog contracted by ATMA was inhibited competitively by hydrogen ion to pH 5.0. Furthermore, the slope of dose-inhibition curve by the hydrogen ion against a certain dose of ATMA was estimated as 1. Also, the slope of does-response curve of ATMA was estimated as 1. From these results, it may be concluded that one molecule of ATMA is inhibited competitively by a hydrogen ion on the ACh receptor. In this case, there is a problem whether the site of the hydrogen ion is the anionic site or the esteratic site, or both sites are affected at the same time. Regarding this point, Bergmann[13] suggested that on the ChE surface both sites are attacked probably by hydrogen ions. In order to solve this problem, the experimental result III was designed according to the procedure reported in the previous paper.[9]

9) M. Kimura : This Bulletin, **7**, 841 (1959).
10) H. Nagano : Kobe-Ikadaigaku-Kiyō, **18**, 153 (1960).
11) M. Fukuya : *Ibid.*, **19**, 133 (1960).
12) Y. Ishida : Yakugaku Zasshi, **81**, 1717 (1961).
13) F. Bergman, *et al.* : J. Biochem., **63**, 684 (1956).

From the estimation that the site of action of hydrogen ion has not stochastically the significant difference from that of hexamethonium ion, and then is independent with organophosphate group, it may be concluded that hydrogen ion attacks to only the anionic site of ACh receptor. Therefore, the apparent dissociation constant pKa obtained in the experimental result IV can be realized as one of the anionic site itself. From the experimental result IV, it was estimated that the pKa of the anionic site is about 5.92. This value exceedingly approaches to the pKa 6.15 in the case of the intestine of mouse.[3] These values are very close to pKa 6.2 of the anionic site of ChE.

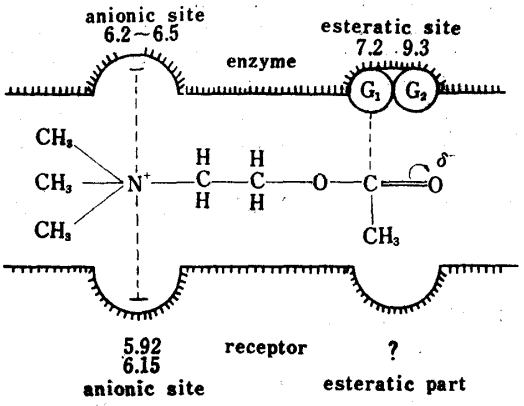


Fig. 8. Hypothetical Picture of Difference between Acetylcholinesterase and Acetylcholine Receptor

On the other hand, since the esteratic site of ACh receptor is not attacked by hydrogen ion, the pKa can not be estimated directly. Therefore, by use of triisopropylphosphate which is supposed to attack the esteratic site of ACh receptor in the previous paper,[3] the association constants between organophosphate group and the esteratic site of ACh receptor and ChE were estimated to compare their sites in the experimental result V and VI. These are brought together for ready comparison in Table III and Fig. 8.

Table III. The Values of $1/K$ between TIP and Esteratic Site of ACh Receptor (AChR) and ChE

	Experiment	Estimation	$1/K$
AChR-TIP	magnus method	parallel line method	1.88×10^{3}
ChE-TIP	manometric method	〃	1.21×10^{2}
〃	〃	double reciprocal method	2.16×10^{2}

These results are as follow; the association constant between organophosphate and the esteratic site of ACh receptor of the intestine of mouse was pK 3.9, and in the case of ChE, pK 2.08 was obtained for ACh receptor by the same design, and also pK 2.33 was obtained by the double reciprocal method. Consequently, in views of the above results, it is difficult to avoid the conclusion that the pK of organophosphate against the esteratic site of ACh receptor is distinctly larger than against that of ChE. Accordingly, the dissociation constant pKa of the esteratic site of ACh receptor seems larger than pKa 7.2 of that of ChE estimated by Wilson & Bergmann.[14]

Secondly, there are some discussions on the mechanism of interaction between their active site and organophosphorylcholine. The experimental result VI gives more distinct inclination on the difference of the effect of organophosphorylcholine against ACh receptor and ChE than a preliminary experiment in the preceding paper.[5] This experimental fact seems to justify the conclusion above described that there is the difference between ACh receptor and ChE, regarding the esteratic site.

Thanks are given to Mr. Tadashi Fujiwara and Mr. Yoshio Kaneko for their assistance in the experimental work.

14) Wilson & Bergmann : J. Biol. Chem., 186, 683 (1950).

Summary

In order to determine whether Acetylcholine (ACh) receptor is identical with Cholinesterase (ChE) or not at the pharmacological or functional level, the mechanism of affinity for the active site on ACh receptor surface is considered with the pharmacological and biochemical method using a usefull tool, organophosphoryl choline. These experiments gave following results :

1) By Magnus method using the rectus abdominis of frog, dose-inhibition curve of amyltrimethylammonium salt (ATMA) and dose-inhibition curve of hydrogen ion against it were observed. From the results, one molecule of ATMA combines with an ACh receptor in the process of the contractile response like the case of ACh, and the site of action of hydrogen ion is located on the anionic site of ACh receptor.

2) The apparent pKa value of the anionic site of ACh receptor is about 5.92 and very close to that of ChE.

3) The two combination constants of organophosphorylcholine with the esteratic site of ACh receptor and of ChE were about 5.53×10^{-4} and $4.63\sim8.27\times10^{-3}$. Therefore, there is the difference between ACh receptor and ChE regarding the esteratic site.

4) From the correlation between anti-ACh and anti-ChE activity of some organophosphorylcholine derivatives, it was indicated that the former increased in the order of $-O-\overset{|}{\underset{|}{P}}=O$, $-O-\overset{|}{\underset{|}{P}}=S$, and $-S-\overset{|}{\underset{|}{P}}=S$, while the latter was the reverse order of them.

5) From the results above mentioned, it may be concluded that ACh receptor is different from ChE molecule.

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2. Kin-ichi Imai and Mikio Honjo : Synthesis of 5-Substituted Pyrimidine Nucleosides.*1

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Until recently, several aldehyde hydrazone derivatives have been reported from different laboratories to be significantly effective as growth retarding agents against some strains of transplantable mammary tumors.[1,2] It has been also reported that a number of 5-substituted pyrimidines (*e.g.*, 5-fluorouracil[3]) are strong antimetabolites for biosynthesis of nucleic acids. In our Research Laboratories, Tanaka and his coworkers[4] synthesized various hydrazone derivatives of *p*-(2,4,6-triamino-5-pyrimidinylazo)benzaldehyde, which were also shown to exhibit similar antitumor activities. Whereas synthesis of 5-substituted aldehyde hydrazone derivatives of the pyrimidines have

*1 Presented at the Kinki Branch Annual Meeting of the Pharmaceutical Society of Japan (Osaka, November, 1963).

*2 Juso-nishino-cho, Higashiyodogawa-ku, Osaka (今井欣一, 本庄美喜男).

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2) R. H. Wiley, R. L. Clevenger : J. Med. Pharm. Chem., **5**, 1367 (1962).
3) C. Heidelberger : "Biological Approaches to Cancer Chemotherapy," 47 (1961). Academic Press, London and New York.
4) K. Tanaka, T. Sugawa, Y. Kuwada, K. Imai, M. Morinaga, J. Watanabe, T. Komeda, T. Usui, H. Yokotani, H. Ito, S. Hemmi, M. Kato, H. Mima, K. Kaziwara : Ann. Rept. Takeda Res. Lab., **22**, 192 (1963).