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153. Masayasu Kimura*[1] : Chemicopharmacological Studies on Antispasmodic Action. XVI.[1] Effect of Hydrogen Ion Concentration on Acetylcholine Receptor.

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In the pharmacological field, there has been only little, if any, interest in the effect of pH on the acetylcholine receptor. Albert[2] drew attention to the relationship between pH and biological activity. It is a known fact, however, that the action of acetylcholine (ACh) is inhibited below pH 7.[3]

In the present work this fact was analyzed experimentally with N-butyltrimethylammonium bromide (BTMA), which has a simpler structure than ACh and which may provide a clue to the estimation of the anionic site in the ACh-receptor.

If the action of hydrogen ion is assumed to be a competitive antagonism to ACh, the hydrogen ion can be treated like atropine was in the previous paper.[4] From the pH-inhibition curves to two kinds of ACh concentration, therefore, two pH values may be estimated, where each ACh response is inhibited to 50%.

The two H^+ concentrations are equal to $[H^+]_1$ and $[H^+]_2$ in equation (3), derived from equation (1) and (2), which were derived from equation (3) in the Part VII of this series.[5] The apparent dissociation constant, pKa, of the ACh receptor can then be calculated from equation (3) in the ratio of ACh concentrations, $r=[A]_1/[A]_2$

$$[H^+]_1=K_H\left(1+\frac{[A]_1}{K_A}\right) \quad (1)$$

$$[H^+]_2=K_H\left(1+\frac{[A]_2}{K_A}\right) \quad (2)$$

$$K_H=\frac{r[H^+]_2-[H^+]_1}{r-1} \quad (3)$$

Method

The method used was the same as in the earlier report,[6] but in this experiment a muscle bath apparatus used was lined with silicone film on the inside wall to prevent alkali from escaping. The bath was filled with a buffer solution made by adding the components shown in Table I to a Tyrode solution free from NaH_2PO_4 and $NaHCO_3$, in order to maintain a constant pH in the bath. In the

Table I. Components of Buffer Solution in 1000 cc. of Modified Tyrode Solution

pH	1% NaH_2PO_4 (cc.)	1% Na_2HPO_4 (cc.)	pH	1% NaH_2PO_4 (cc.)	1% Na_2HPO_4 (cc.)
7.4	0.25	6.00	5.8	6.00	0.80
6.7	4.50	5.50	5.6	6.00	0.35
6.4	6.50	4.50	5.4	6.00	0.15
6.1	6.00	1.75	5.2	8.00	0.10
6.0	6.00	1.50			

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1) Part XV : This Bulletin, **6**, 716(1958).
2) A. Albert : Pharmacol. Rev., **4**, 136(1952).
3) R. Chihara : Japan. J. Pharmacol., **51**, 515(1955).
4) K. Takagi, M. Kimura : This Bulletin, **5**, 440(1957).
5) K. Takagi, *et al.* : *Ibid.*, **5**, 247(1957).
6) K. Takagi, M. Kimura : *Ibid.*, **4**, 444(1956).

presence of a hydrogen carbonate, a stream of air removes CO_2 and pH of the bath solution shifts to the alkaline side.

Results

I. Dose-Response Curve of BTMA—This experiment was conducted to prove that BTMA may be substituted for ACh. Doses of BTMA were divided into six levels from $5.1\times10^{-6}M$ to $1.13\times10^{-4}M$, with $1.06\times10^{-3}M$ as the maximum contracting dose.

In Table II are given the result of observations using 10 mice and the graph shown in Fig. 1 gives the formula, $Y=1.224X+13.369$.

Table. II. Data of Dose-Response Curve of BTMA

BTMA (M)	Repetition	Response (%)	BTMA (M)	Repetition	Response (%)
5.1×10^{-6}	10	18.2	4.08×10^{-5}	10	72.7
1.02×10^{-5}	10	31.4	8.16×10^{-5}	10	86.2
2.04×10^{-5}	10	53.9	1.13×10^{-4}	10	94.0

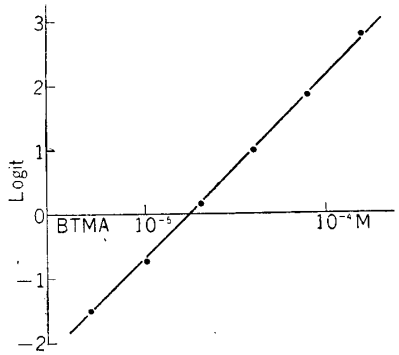

Fig. 1. Logistic Regression Line of N-Butyltrimethylammonium Bromide (BTMA) on Small Intestines of Mice

From the analysis of variance of the results $s^2=0.007174$ and $D_0^2=0.00093$ were calculated by the graphic method[7] and then the t-test showed that the slope of BTMA as well as ACh did not deviate significantly from the value of 1.

II. Effect of pH on Dose-Response Curves of BTMA—A split-plot design was adopted in this experiment using 5 mice each for a given pH value, totaling 35 mice. The results are shown in Fig. 2.

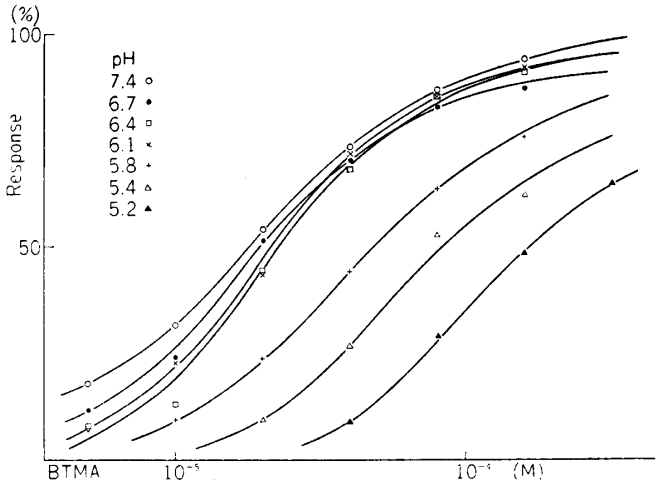

Fig. 2. Effect of Lowering pH on Dose-Response Curves of N-Butyltrimethylammonium on Small Intestines of Mice

From Fig. 2 it is apparent that a hydrogen ion concentration of over pH 6.0 has little effect on the action of BTMA, but below pH 6.0 it has a marked inhibitory effect. A concentration below pH 5.0 caused intestinal muscles to be irreversibly inhibited.

7) K. Takagi, *et al.*: Yakugaku Zasshi, **76**, 1191(1956).

III. Effect of pH on the Maximum Contractile Responses of BTMA and ACh—In Table III it is seen that the maximum responses are not depressed by H^+ above pH 5.5 and it is difficult to avoid concluding that the hydrogen ion acts competitively on BTMA and ACh, at least, at a pH above 5.5.

Table. III. Effect of pH on Maximum Responses of BTMA and ACh

pH	BTMA ($5.1\times10^{-3}M$) (%)	ACh ($5.5\times10^{-4}M$) (%)	pH	BTMA ($5.1\times10^{-3}M$) (%)	ACh ($5.5\times10^{-4}M$) (%)
7.4	100.0	100.0	5.5	98.2	97.3
6.1	103.4	98.1	5.2	61.0	78.6
5.8	98.2	99.8			

IV. Estimation of the Apparent pKa Value between ACh Receptor and Hydrogen Ion—In order to obtain two dose-inhibition curves in the range of pH 5.6 and 6.0, the next step was performed on (a) BTMA ($6.38\times10^{-6}M$, $2.55\times10^{-5}M$) with 10 mice, and (b) ACh ($1.1\times10^{-7}M$, $4.4\times10^{-7}M$) with 7 mice. Results are shown together for ready comparison in Figs. 3 and 4.

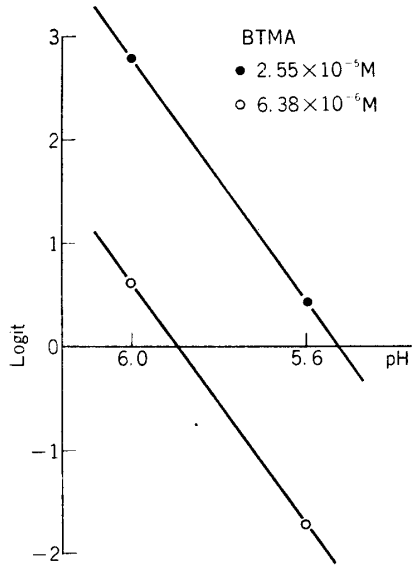

Fig. 3. Dose-Inhibition Curves of Hydrogen Ion to N-Butyltrimethylammonium Bromide

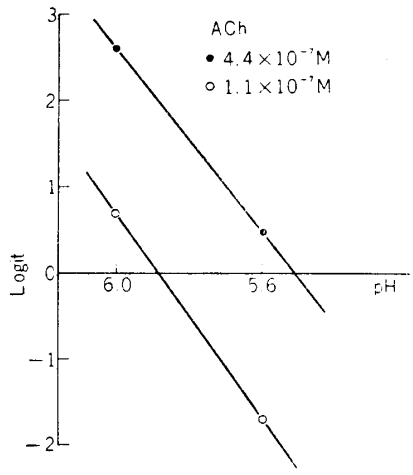

Fig. 4. Dose-Inhibition Curves of Hydrogen Ion to Acetylcholine

In Table IV the results of calculations made for estimating pKa of the anionic site in the ACh receptor are given.

Table IV. Estimation of pKa Value of the Anionic Site (R^-) in ACh Receptor

BTMA		Concentration of agonist	ACh	
$2.55\times10^{-5}M$	$6.38\times10^{-6}M$		$4.4\times10^{-7}M$	$1.1\times10^{-7}M$
5.52	5.89	pH of 50% inhibition	5.51	5.89
−2.67		slope	−2.53	
0.711×10^{-6}		apparent K_a of R^-	0.689×10^{-6}	
6.15		pKa of R^-	6.16	

The value of the apparent K_a given in Table IV was calculated as $n=1$ in the equation (3), as it was considered that the slope of pH-inhibition curves, about 2.5~2.7, is independent of the reaction order itself.

Discussion

In studies on the effect of pH on ACh-receptor complex, it is well known that the structure of ACh as well as the function of the cell are hardly changed by a pH between 5 and 8.[9~10] A discussion may therefore be made on the condition when hydrogen ions

8) I. B. Wilson, F. Bergmann : J. Biol. Chem., **185**, 683(1950).
9) R. L. de No : Studies from the Rockefeller Institute for Medical Research, **31**, 132(1947).
10) A. J. Clark : J. Physiol., **64**, 123(1927).

of over pH 5.5 affect only the ACh-receptor. The disadvantage of adding HCl directly into Tyrode solution in a muscle bath is very probably due to the fact that the chemical reaction of $NaHCO_3$ with HCl changes pH to the alkaline side. Even the presence of hydrogen carbonate makes it difficult to maintain the pH of the bath solution constant. According to recent communications[11,12] it has been tentatively suggested that the receptor is a kind of protein or lipoprotein.

In the electrostatic connection of N-butyltrimethylammonium ion ($BTMA^+$) and hydrogen ion (H^+) with the anionic site (R^-) in the ACh receptor, H^+ can be treated as a competitive antagonist when it is supposed that the $R^-—BTMA^+$ complex comes into active process, while the R—H complex comes into inactive process in equation (4).

$$R^- + BTMA^+ = R^- — BTMA^+ \quad (4\text{-}1)$$

$$R^- + H^+ = R — H \quad (4\text{-}2)$$

From equation (3), the apparent dissociation constant of R—H was experimentally estimated as pKa=6.15 using BTMA, and as pKa=6.16 using ACh. In addition to these calculated results, a comparison between ACh and BTMA may be made as follows: (1) Both agree on the slope of dose-inhibition curve by H^+, (2) neither has different response ratio at pH 5.2 with their high concentration, and (3) the slope of the dose-response curve of BTMA was estimated as 1, as that of ACh.

From these observations it was concluded that the cationic head in ACh as well as in BTMA may make an attack on the same anionic site in the ACh receptor and thereby be inhibited by H^+. However, it is not sufficient to conclude on the nature of anionic site from the observed pKa, though that of the ester of diphosphate is near 6.2.[13]

To consider the ACh-receptor itself, there is an important problem that confronts the relationship between the ACh-receptor and ACh-esterase. No experiments have yet been made which allows one to evaluate the probability that the ACh-receptor is ACh-esterase itself, but it has, however, been suggested that the ACh-receptor is identical with its esterase.[14] Recently Nachmansohn[15] stated that the difference between them may not be dependent on their active sites, but on the part of a protein. His considerations are supported by the present experimental results that pKa of anionic site in the ACh-receptor is 6.15~6.16, which agrees very closely with pKa 6.2 of anionic site in the ACh-esterase reported by Wilson and Bergmann.[13] However, there is as yet not sufficient evidence to give an accurate solution to this problem.

The author is indebted to Prof. K. Takagi for suggesting this investigation as well as for constant guidance during the course of the work.

Summary

(1) A modified salt solution, which does not contain sodium hydrogen carbonate, was used in this experiment to study the effect of pH on the small intestines of mice.

(2) The dose-contraction curve of N-butyltrimethylammonium bromide obeyed Clark's equation and the slope was 1.2. The curve was inhibited competitively in pH range between 6.0 and 5.5.

(3) From the interaction between N-butyltrimethylammonium bromide and H^+, the pKa of the anionic site of ACh-receptor was estimated as 6.15 and as 6.16 in the case of ACh and H^+. These values are very close to pKa 6.2 of the anionic site of ACh-esterase, estimated by Wilson and Bergmann.

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11) J. H. Welsch : Bull. Johns Hopkins Hosp., **83**, 568(1949).
12) I. B. Wilson : "Currents in Biochemical Research," Ed. D. E. Green, 642(1956).
13) F. Bergmann, *et al.* : Biochem. J., **63**, 684(1956).
14) A. O. Zupancic : Acta Physiol. Scand., **29**, 63(1953).
15) D. Nachmansohn : "Currents in Biochemical Research," Ed. D. E. Green, 628(1956).