U.D.C. 615.782-015.25

44. Keijiro Takagi and Issei Takayanagi: Chemicopharmacological Studies on Antispasmodic Action. VII. Antagonism to Acetylcholine tested with Rectus Abdominis of Frog.

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As rectus abdominis of frog is very sensitive to acetylcholine (ACh) and no spontaneous movement is producible, it was used for studying the reaction mechanism between ACh and its receptor, and antagonistic action of atropine or *d*-tubocurarine to ACh.

Quantitative studies of the reaction between ACh and its receptor, and of the competitive inhibition by atropine have been made by Clark[1] and others.[2]

In the previous paper[3] we discussed the equation (1) for the action of ACh.

$$\frac{e^{X_A}}{K_A} = \frac{y}{y'-y} \tag{1}$$

X_A=log of molecular concentration of ACh

K_A=dissociation constant of the receptor to ACh

y' =maximum response y =response at X_A

Atropine, a typical antiacetylcholine agent, is thought to inhibit competitively the action of ACh. As for theoretical interpretation, Gaddum[4] presented formula (2) for an inhibition by atropine.

$$\frac{e^{X_A}}{K_A} = \left(1 + \frac{e^{nX_B}}{K_B}\right) \frac{y}{y'-y} \tag{2}$$

X_A=log of molar concn. of ACh X_B=log of molar concn. of atropine

n =no. of atropine molecules combining with the same ACh receptor

K_A, K_B=dissociation const. of ACh receptor and atropine receptor combination

y' =maximum response y =response at X_A with X_B

The mode of action of antagonist B is assumed in the equation (2) that B combines with the same receptor as ACh and that the combination can not initiate the response like ACh.

In the presence of the antagonist B the dose-response curve of ACh moves parallel to a higher concentration.

From the eqation (2), equation (3) was derived.

$$\frac{e^{nX_B}}{K_B} = \left(1 + \frac{e^{X_A}}{K_A}\right) \frac{y''-y}{y} \tag{3-1}$$

$$y'' = \left(\frac{e^{X_A}}{K_A} \Big/ 1 + \frac{e^{X_A}}{K_A}\right) y' \tag{3-2}$$

The equation (3) shows an inverse logistic sigmoid. We tried to prove statistically, by the method previously reported by us,[3] that the concentration-acton relationship would obey the equation mentioned above in the excised muscle preparation of frog rectus abdominis.

Method

(1) Healthy female frog (*R. nigromaculata*) weighing 25~45 g. were used. No consideration was

* Hongo, Tokyo (高木敬次郎, 高柳一成).

1) A. J. Clark: J. Physiol., **61**, 530(1926).
2) H. Matumoto: Medical Sience, **6**, 3, 113(1954).
3) K. Takagi, *et al.*: This Bulletin, **4**, 444(1954).
4) J. H. Gaddum: J. Physiol., **89**, 7p(1937).

given to the different seasons. The method of making an isolated muscle preparation and recording contractive responses was the same as usual.[5]

The fluid, in which the muscle was bathed, was replaced by a solution containing ACh. The maximum height produced by that concentration of ACh was measured as the response. After each ACh solution was replaced with Ringer solution, the muscle was washed three or more times over a period of at least 7 mins. or until the complete relaxation to the original level occurred. The percentage contraction relative to the maximum contraction (Ho) produced by 2×10^{-4} g./cc. of ACh was preferred as the response metameter.

(2) In the presence of an antagonist, the muscle was kept in the Ringer solution containing the antagonist for 5 mins. and the solution was replaced by the antagonist-Ringer solution containing ACh.

(3) In the experiments of eserinized muscle, the muscle was kept in the Ringer solution containing the eserine 2×10^{-5} g./cc. for a definite period (1.0 hr. or 2.0 hrs.) before the experiment.

(4) In constructing the dose-response curves of ACh, atropine, and *d*-tubocurarine, various doses of each drug were applied in the same preparation at random.

(5) After the response to the concentrations of each ACh in the absence of antagonist was determined, the experiment with an antagonist was carried out.

(6) The graphic method described in the previous reports[6,7] was used to treat the experimental data. The doses exhibiting 10 to 90% responses were adopted for the calculation.

(7) For assying parallelism of several logistic regression lines, most probable lines are drawn provisionally through several sets of data.

From the i-th line, weighing coefficients W_i and slope bi will be estimated and the sum of squares for regression B_i^2 will be calculated according to the method previously described by us.[7]

$$B_i^2 = b_i^2[S_{x^2}]_i \tag{4}$$

The combined slope b_c is given as follows:

$$b_c = \sum b_i \cdot [S_{x^2}]_i / \sum [S_{x^2}]_i \tag{5}$$

$$B_c^2 = b_c^2 \cdot \sum [S_{x^2}]_i \tag{6}$$

The sum of squares accounted for deviation from parallelism is then given:

$$D_p^2 = \sum_{i=1}^{k} B_i^2 - B_c^2 \quad (\text{d.f.} = k - 1) \tag{7}$$

Experimental Results

1) Response to ACh—Because the variance of the lowest and highest doses are smaller than that of the other concentrations, the intermediate 6 concentration levels were used to calculate the ACh dose-response curve (Fig. 1).

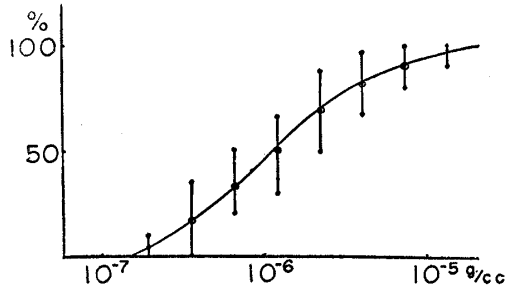


Fig. 1.
ACh Dose-Response Curve ($H_0=2\times10^{-4}$ g./cc.)
The vertical lines indicate the range of responses.
Responses at ⊙ were used for calculation.

As a result of calculation this may be considered as a logistic curve, of which the slope is 1.051. (Table I).

2) Response to ACh in the Presence of Eserine—ACh dose-response curves in the presence of eserine were sigmoid curves, but the range of effective concentrations was narrower, and the maximum contraction was greater than those without eserine.

When the muscle was eserinized for 1 hr., the slope of ACh dose-response curve increased to 1.651 (Fig. 2 and Table II) and in eserinizing for 2 hrs., n increased further to 2.238 (Fig. 2 and Table II).

5) E.F. van Maanen: J. Pharmacol. Exptl. Therap., **99**, 255(1950).
6) K. Takagi, *et al.*: J. Pharm. Soc. Japan, **76**, 1187(1956).
7) K. Takagi, *et al.*: *Ibid.*, **76**, 1196(1956).

Table I. ACh Dose-Response Curve

Nature of variation	d.f.	Mean square
Deviation from regression	4	0.0043
Between doses[a)]	5	0.0463*
Between animals	9	0.4178**
Error	45	0.0065
Total	59	

$b=1.051$, $t_0=1.023$[b)] as $\beta=1$ ($t=2.776$, d.f.$=4$, $P=0.05$)

In all the Tables in this report,

a) ⊙ in the corresponding figures were used for this calculation.

b) $t_0=(b-\beta)/\sqrt{V(b)}$.[7)]

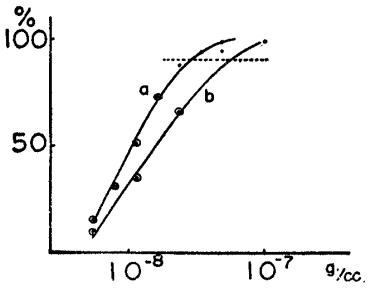


Fig. 2.
ACh Dose-Response Curve in the Presence of Eserine

Curve a=eserinized for 1 hr.
Curve b=eserinized for 2 hrs.
The dotted line is the maximum without eserine.

Table II. ACh Dose-Response Curve in the Presence of Eserine

Curve a=eserinized for 1 hr.

Nature of variation	d.f.	Mean square
Deviation from regression	2	0.0010
Between doses[a)]	3	0.0498*
Between animals	5	0.2664**
Error	15	0.0073
Total	23	

$b=1.651$, $t_0=1.023$[b)] as $\beta=1.5$ ($t=4.303$, d.f.$=2$, $P=0.05$)

Curve b=eserinized for 2 hrs.

Nature of variation	d.f.	Mean square
Deviation from regression	1	0.0046
Between doses[a)]	2	0.0244*
Between animals	4	0.2659**
Error	8	0.0043
Total	14	

$b=2.238$, $t_0=1.297$[b)] as $\beta=2$ ($t=12.71$, d.f.$=1$, $P=0.05$)

3) **Action of Atropine**—ACh dose-response curve moved parallel to higher concentration of ACh by atropine (Fig. 3 and Table III) (Fig. 4 and Table IV).

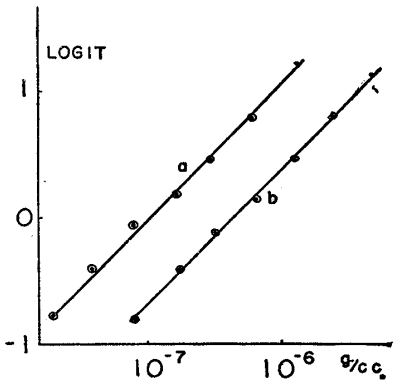


Fig. 3.
Movement of ACh Dose-Response Curve by Atropine (Logit)

Curve a=ACh only
Curve b=with 10^{-4} g./cc. of atropine

Table III. Movement of ACh Dose-Response Curve by Atropine

Nature of Variation	d.f.	Mean square
Deviation from regression	a) 4	0.0016
	b) 4	0.0050
Parallelism	1	0.0069
Between doses[a)]	11	0.0243*
Between animals	5	0.7593**
Error	55	0.0047
Total	71	

slope of a) $b=0.9750$, $t_0=0.3035^{b)}$ as $\beta=1$ ($t=2.776$, d.f.$=4$, $P=0.05$)
〃 b) $b=0.9126$, $t_0=1.670^{b)}$ as $\beta=1$ (〃)

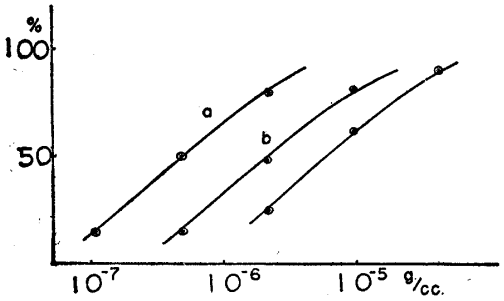


Fig. 4.
Movement of ACh Dose-Response Curve by Atropine

Curve a = ACh only
Curve b = with 10^{-4} g./cc. of atropine
Curve c = with 2×10^{-4} g./cc. of atropine

Table IV. Movement of ACh Dose-Response Curve by Atropine

Nature of variation	d.f.	Mean square
Deviation from regression	a) 1	0.0013
	b) 1	0.0061
	c) 1	0.0070
Parallelism	2	0.0019
Between doses[a)]	8	0.0498*
Between animals	5	0.6847**
Error	40	0.0069
Total	53	

slope of a) $b=0.9808$, $t_0=0.1201^{b)}$ as $\beta=1$ ($t=12.71$, d.f.$=1$, $P=0.05$)
〃 b) $b=1.1387$, $t_0=1.130^{b)}$ as $\beta=1$ (〃)
〃 c) $b=1.2032$, $t_0=0.7431^{b)}$ as $\beta=1$ (〃)

Dose-response curve of atropine to a definite concentration of ACh was the inverse sigmoid curve, of which n is estimated as 1.5 (Fig. 5 and Table V).

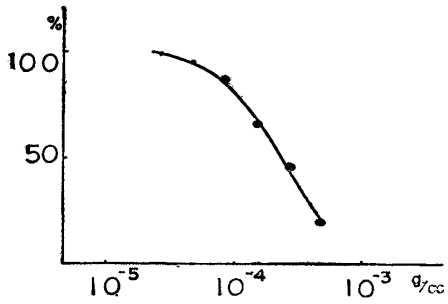


Fig. 5.
Dose-Response Curve of Atropine to ACh (10^{-5} g./cc.)

Ordinate = contractive response to ACh
Abscissa = $\log_{10}$ (concentration of atropine)

Table V. Dose-Response Curve of Atropine

Nature of Variation	d.f.	Mean square
Deviation from regression	2	0.0020
Between doses[a)]	3	0.0248*
Between animals	9	0.3200**
Error	27	0.0049
Total	39	

$b=1.503$, $t_0=0.1201^{b)}$ as $\beta=1.5$ ($t=4.303$, d.f.$=2$, $P=0.05$)

4) Action of *d*-Tubocurarine—ACh dose-response curve also moved parallel by *d*-tubocurarine (Fig. 6). This may be considered as two logistic curves which are parallel to each other and the slope in both was 1 (Table VI).

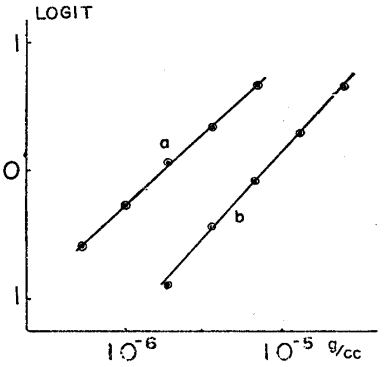


Fig. 6.
Movement of ACh Dose-Response Curve by *d*-Tubocurarine

Curve a = ACh only
Curve b = with 10^{-5} g./cc. of *d*-tubocurarine

TABLE VI. Movenemt of ACh Dose-Response Curve by *d*-Tubocurarine

Nature of variation	d.f.	Mean square
Deviation from regression	a) 3	0.0051
	b) 3	0.0036
Parallelism	1	0.0127
Between doses[a)]	9	0.0621*
Between animals	4	0.8792**
Error	36	0.0054
Total	49	

Slope of a) $b=1.071$, $t_0=0.679^{b)}$ as $\beta=1$ ($t=3.182$, d.f.$=3$, $P=0.05$)
〃 b) $b=1.176$, $t_0=1.176^{b)}$ as $\beta=1$ (〃)

Dose-response curve of *d*-tubocurarine to a definite concentration of ACh is an S-shaped curve, whose range is about twice as broad as that of ACh. As the result of calculation, this may be regarded as a logistic curve of which n is 0.55 (Fig. 7 and Talbe VII).

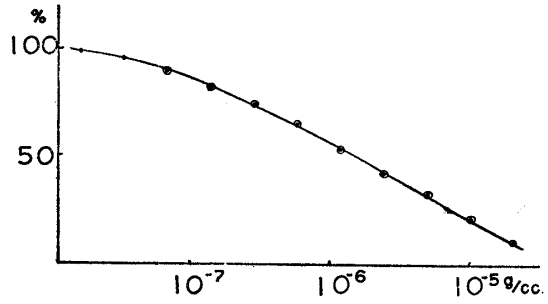


Fig. 7.
Dose-Response Curve of *d*-Tubocurarine to ACh (10^{-6} g./cc.)

Ordinate = contractive response by ACh
Abscissa = $\log_{10}$ (concentration of *d*-tubocurarine)

TABLE VII. Dose-Response Curve of *d*-Tubocurarine

Nature of variation	d.f.	Mean square
Deviation from regression	7	0.0531
Between doses[a)]	8	0.1974*
Between animals	12	0.6781**
Error	96	0.0960
Total	116	

$b=0.5501$, $t_0=0.5891^{b)}$ as $\beta=0.5$ ($t=2.365$, d.f.$=7$, $P=0.05$)

Discussion

1) Response of ACh

Recently, it was reported by Chihara[8)] that ACh dose-response curve obeyed the law

8) R. Chihara: Japan. J. Pharmacol., **51**, 531(1955).

of mass action, which Clark[1] had pointed out, and that the slope was 1.57±0.08.

The present result agrees with that of Clark and it may be concluded that the reaction is governed by the law of mass action, and that one molecule of ACh must react with one receptor.

2) For the influence of eserine it was reported by Matsumoto[2] and Chihara[8] that on the rectus abdominis of a frog, ACh dose-response curve using a lower concentration of eserine shifted to a lower concentration of ACh and that in a higher concentration, the inhibition of maximum contraction was attended with the parallel movement of the curve. It was reported by Cavanaugh, *et al.*[9] that the number of molecules of ACh reacting with its receptor was found to be two in the presence of eserine. Ogyu's report[10] suggested that slope-coefficient of ACh in the absence of eserine was not similar to that of ACh in the presence of eserine with the probit-transformation in the study on curare-like action of quaternary bases on rectus abdominis of a frog.

Now, it can be concluded from our results that the maximum contraction is clearly higher than that in the absence of eserine. From our experiment also, it can be recognized that the longer the duration of eserinizing, the larger is the slope, because the action of eserine should not be limited to its anticholinesterase action to muscles, it is questionable to deduce the reaction order from the results stated above.

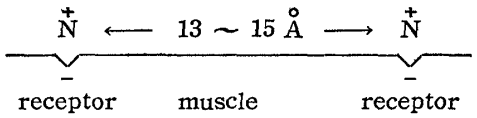
3) **Action of Atropine**

A competitive antagonism between ACh and atropine was pointed out by Clark[11] and Gaddum.[4] Clark reported that in the case of the action of atropine on the rectus abdominis of a frog, n was 1.5, and that Gaddum's equation (2) agreed to a fair extent. As our results also agree with that of the past, it is sure that antagonism is competitve. From the dose-inhibition curve on various concentrations of atropine against a definite concentration of ACh, the number of atropine molecules, n, combining with ACh-receptor can be obtained according to equation (3), derived in turn from equation (2), and n proved to be 1.503, which deviates not significantly from 1.5 at 95% probability level (Table V).

The reason, why n is 1.5, need be studied in future. In the case of antagonism between ACh and atropine on the rectus abdominis, higher concentration of atropine than that on smooth muscle was needed, and its action should be antinicotinic. Therefore, rectus abdominis of a frog cannot be applied to the assay for antimuscarinic action on smooth muscles.

4) **Action of *d*-Tubocurarine**

In regard to *d*-tubocurarine, it was reported by van Maamen[5] and by Ogyu[10] that the antagonism is considered competitive like atropine and n is 0.5 by the present results. It can therefore be considered that two ammonium nitrogens in *d*-tubocurarine molecule antagonize two molecules of ACh.



This fact indicates that neuromuscular blocking action of *d*-tubocurarine should be purely competitive, and that the drug should not provoke any positive physiological changes there.

On the other hand, it was reported by Taylor, *et al.*[12] that in the study of the mode of action of curare alkaloids on neuromuscular transmission in rat diaphragm preparations

9) D. J. Cavanaugh, *et al.* : Arch. intern. Pharmacodynamie, **100**, 68(1954).
10) K. Ogyu, *et al.* : Japan. J. Pharmacol., **51**, 209(1955).
11) A. J. Clark : J. Physiol., **61**, 547(1926).
12) D. B. Taylor, *et al.* : J. Pharmacol. Exptl. Therap., **103**, 382(1951).

the response of *d*-tubocurarine to a definite electric stimuli was an S-shaped curve having smaller range between 0.1~0.4 mg./cc. From this fact, it is considered that there should be a different mechanism of transmission between rectus abdominis stimulated by ACh and the neuromuscular preparation of a rat diaphragm stimulated electrically.

Summary

It was concluded statistically on the contraction of rectus abdominis of a frog that:

1) The reaction between ACh and its receptor in the muscle obeyed Clark's formula and the reaction order was 1, that is, they react with one molecule each.

2) Reaction mechanism between ACh and its receptor was different in the presence of eserine, from that in its absence.

3) Antagonism of atropine and *d*-tubocurarine to ACh obeyed Gaddum's formula as competitive.

4) One molecule of *d*-tubocurarine antagonized two molecules of ACh; two ammonium nitrogens in *d*-tubocurarine antagonized one molecule of ACh each.

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45. Daisuke Satoh, Takayuki Wada, Hiroshi Ishii, Yohko Oyama, and Tamotsu Okumura : Studies on Digitalis Glycosides. VII.[1] Gitoroside,* Digitalonin, and Gitoxin Pentaacetate.

(*Research Laboratory, Shionogi & Co., Ltd.***)

The pale yellowish brown substance, m.p. 210~216°, separated from the mother liquor of gitoxin, described in the previous paper,[1] gave a colorless powder, m.p. 213~216°, after purification with ethyl acetate. This substance showed a similar coloration with gitoxin in the Legal and the Keller-Kiliani reaction. However, the solubility and the Rf value on paper chromatogram of this substance differed from those of gitoxin. U.V. λ_{max}^{EtOH} 219 mμ (log ε 4.15). $[\alpha]_D^{26}$ +24.9°(c=0.4985, EtOH). Analytical values corresponded to $C_{29}H_{44}O_8 \cdot 1\frac{1}{2} H_2O$ and had no methoxyl nor acyl group.

The acetate formed a colorless powder, m.p. 128~131°, and result of paper chromatography indicated the unity of this substance. Analyses gave values which agreed with those of a triacetate, $C_{35}H_{50}O_{11} \cdot H_2O$.

Hydrolysis of this glycoside under mild conditions gave an aglycone as colorless needles, m.p. 223~225°, whose analytical values corresponded to $C_{23}H_{34}O_5$. Mixed fusion of this aglycone with authentic sample of gitoxigenin, m.p. 224~227°, showed no depression of the melting point and the Rf value of this aglycone agreed with that of gitoxigenin.

Since the paper chromatogram of the sugar moiety of this glycoside gave only one spot by the Keller-Kiliani reaction and by aniline hydrogen phthalate, it is obvious that there was one kind of 2-desoxysugar. On comparison of Rf values, the sugar moiety was shown to be *d*-digitoxose. A crystalline sugar, m.p. 103~105°, was obtained by the vacuum sublimation of the syrupy sugar and the mixed fusion of this sugar with authentic

* A brief summarized report on gitoroside was published as a Communication to the Editor in J. Pharm. Soc. Japan, **76**, 1334(1956).

** Imafuku, Amagasaki, Hyogo-ken (佐藤大助, 和田敬之, 石井 宏, 尾山蓉子, 奥村 保).

1) Part VI : D. Satoh, H. Ishii, Y. Oyama, T. wada, T. Okumura : This Bulletin, **4**, 284(1956).