U. D. C. 615.711.76-092.259

83. Keijiro Takagi and Masayasu Kimura : Chemicopharmacological Studies on Antispasmodic Action. V.[1] Fundamental Conditions of Bioassay of Acetylcholine and Atropine on the Isolated Small Intestines of Mice.

(*Pharmaceutical Institute, Medical Faculty, University of Tokyo**)

An evaluation of recent reviews[2,3] has suggested that many synthetic studies of antispasmodic agents had been tried for the purpose of discovering some drugs with ideal antispasmodic action. The methods, however, are seemingly of empirical nature in designing new compounds, with rather a little consideration on the mode of blocking action upon a hypothetic receptor surface.

Our present investigation, on the other hand, was initiated to determine this relationship in order to create a new design of chemical structure with the most effective antispasmodic activity. This paper is devoted primarily in establishing a fundamental condition for the bioassay of acetylcholine (ACh) and atropine (Atr).

Bentley and Shaw[4] referred to various spastic preparations used for the assay of ACh, which are used in our experiment as a spasmogenic agent. In fact, many examples of using isolated small intestines of guinea pigs or rabbits are found, but we have as yet very little information dealing with mice in this kind of experiment. We now used the small intestines of mice because this animal was found the most convenient for our statistical experiments. Therefore, before the bioassay of ACh and Atr, some characteristics of isolated small intestines of mice were examined.

Method

The experimental method used in this study has been fully described elsewhere by Takagi *et al.*[5] and only a brief outline need be given here.

Young mice, weighing 13～18 g., were used. A piece of the intestine, 15～25 mm. long was cut off and two of these segments were suspended together in a muscle bath containging Tyrode solution at 26°. The bath was filled exactly to 30 cc. and the content was constantly agitated by bubbling air through the solution. The tissue was always rested for at least 10 mins. with frequent changes of Tyrode solution and a constant response to ACh was established before the assay was commenced. After exposure to the agents, the Tyrode solution was removed and the excised organ was washed by refilling three or more times between each observation. Then ACh appropriately diluted to 1 cc. was added directly to the bath in the fixed position in 20 secs. Atr was also added previously in the same way before ACh and the next addition of ACh was made within the following 120 secs.

Changes in the rate of contraction were recorded kymographically. The contractile response was shown by the maximum height appearing on the drum within 60 secs. from the start of adminisration, while the activity of Atr was shown as the rate of contraction inhibition.

Results

Fig. 1 is a record of observations in one intestine. Each concentration of the drug was applied in a randomized order.

(1) Optimal temp. of a muscle bath: Observations using 4 mice with $1.1\times10^{-6}M$ of ACh, were made at four temp. of 20°, 26°, 32°, and 38°, and the results are shown in Tables I and II.

(2) Mode of administration of drug solutions into the bath: Since the mode of administration affects responsive sensitivity, the drug solution was introduced in a fixed position using an apparatus shown in Fig. 3. Into a narrow bore of a small glass tube (a), a syringe needle was inserted, and

* Hongo, Tokyo (高木敬次郎, 木村正康).

1) Part IV. K. Takagi *et al.* : J. Pharm. Soc. Japan, **75**, 566(1955).
2) R. B. Burtner in "Medicinal Chemistry." Chief Editor : C. M. Suter. **I**, 151(1951).
3) K. Takagi, Y. Kasuya : Medical Science, **5**, 213(1954).
4) G. A. Bentley, F. H. Shaw : J. Pharm. Exptl. Therap., **106**, 193(1952).
5) K. Takagi, Y. Kasuya : J. Pharm. Soc. Japan, **73**, 541(1953).

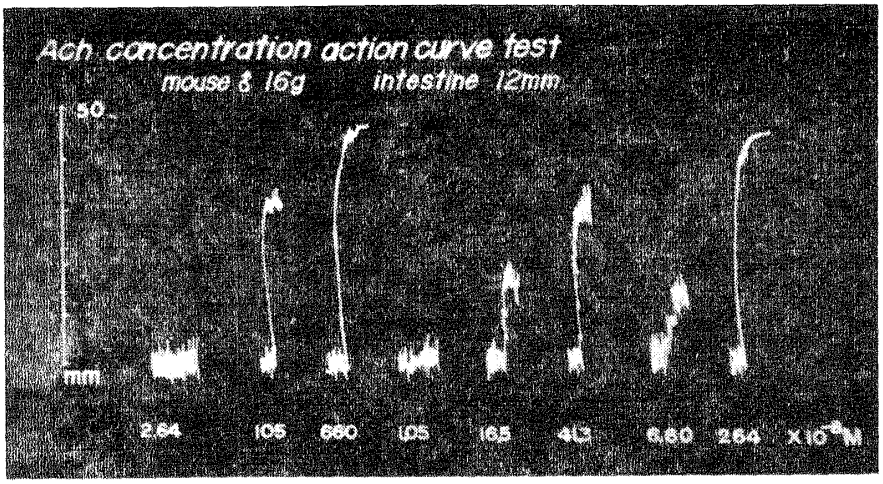

Fig. 1. An Example of Observations with the Isolated Small Intestine of Mouse

TABLE I. Effect of Temperatures upon the Contractility of Small Intestines of Mice (mm.)

		20°	26°	32°	38°
A_1	I_1	24.5	23.0	18.0	17.5
	I_2	24.0	19.5	21.0	13.5
A_2	I_1	45.0	45.0	58.0	54.0
	I_2	46.0	44.0	54.0	54.5
A_3	I_1	7.5	10.5	7.0	4.5
	I_2	7.0	13.5	5.5	6.5
A_4	I_1	54.5	54.0	42.0	26.5
	I_2	53.5	58.0	53.5	28.5
Total		262.5	262.5	259.5	203.5
No.		8	8	8	8
Means		32.8	32.8	32.4	25.4

TABLE II. Analysis of Variance for the Data of Table I

Adjustment for Mean 30504.5

Nature of Variation	d.f.	Sum of Squares	Mean Square
Temperature	3	316.13	105.4
Animals	3	988.29	329.4
T × A	9	6769.46	752.2
Intestines	16	3260.12	203.8
Total	31	11334.00	

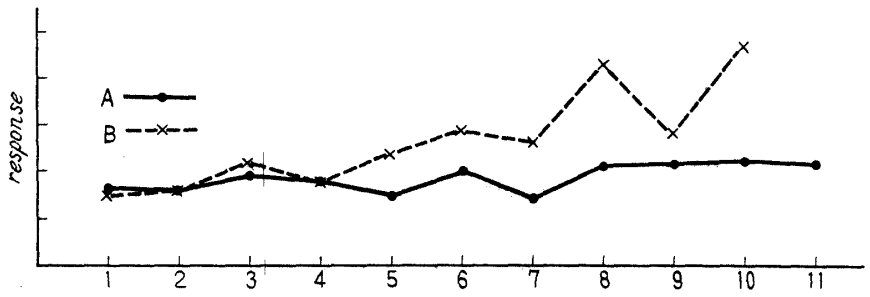

Fig. 2. Variation of the Data in Table III

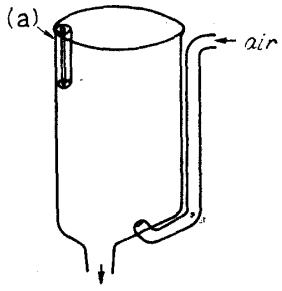

Fig. 3. Apparatus of the Bath with a Small Glass Tube (a)

TABLE III. Variation of Responses in the Two Methods of Administration (mm.)

	1	2	3	4	5	6	7	8	9	10	11	Total	Means
A	16	16	19	18	15	20	14	21	21	22	21	203	18.5
B	15	16	22	18	24	29	26	43	28	47		268	26.8

$SA = 78.8$ $U_A^2 = 7.88$ $\therefore$ $U_B^2/U_A^2 = 14.18 \gg F_{10}^9(0.01)\,4.95$

$SB = 1041.6$ $U_B^2 = 115.73$

a given volume of the drug solution was added through it at a definite time. This fixed method (A) was compared with the usual arbitrary method (B) that did not use the glass tube (a), in the same final concentration of $2.2\times10^{-6}M$ ACh. This is shown in Table III and Fig. 2.

(3) Test of linearity of ACh dose-response curve: These observations were made to find the linear portion out of a dose-response curve in the approximate range of concentrations, 2.8×10^{-8} to $2.8\times10^{-6}M$, which were found by the previous experiments.

Doses were divided into four degrees, i.e. 1.38×10^{-7}, 2.75×10^{-7}, 5.5×10^{-7}, and $1.1\times10^{-6}M$ which were applied twice on the same intestine, and $2.75\times10^{-6}M$ was appointed as the maximum contractile dose. The percentage contraction relative to the maximum height was selected as a response metameter. Exercises of the same dose series were repeated on several pairs of intestines and the results obtained for these values are given in Tables IV to VI and in Fig. 4.

Table IV. Contraction Ratio of Small Intestines of Mice at Four Dosage Levels of ACh

	$\times10^{-7}M$	1.38	2.75	5.5	11
A_1	I_1 i	26.6	54.5	63.3	79.8
	I_1 ii	36.8	77.3	72.7	100.0
	I_2 i	43.3	62.8	65.7	91.1
	I_2 ii	28.4	76.1	70.2	80.6
A_2	I_1 i	20.0	30.0	66.7	76.8
	I_1 ii	23.0	46.7	63.4	76.8
	I_2 i	12.0	22.0	50.0	70.0
	I_2 ii	18.0	30.0	56.0	76.2
A_3	I_1 i	13.9	45.9	59.7	83.4
	I_1 ii	15.3	47.8	65.3	75.0
	I_2 i	19.1	45.3	69.0	93.0
	I_2 ii	26.2	45.3	81.0	83.5
A_4	I_1 i	26.2	39.4	52.0	67.2
	I_1 ii	16.4	47.6	50.9	65.5
	I_2 i	46.0	48.3	63.2	81.6
	I_2 ii	34.5	55.2	72.5	80.5
Total		406.0	773.7	1021.5	1281.0
Mean		25.4	48.5	65.1	80.1

Table V. Analysis of Variance for the Data of Table IV

Adjustment for Mean		189453.44		
Nature of Variation	d. f.	Sum of Squares	Mean Square	
Regression	1	25784.18		
Deviation from regression	2	238.30	119.15	
Doses	3	26022.48		
Intestines	7	4044.46		
Animals	3	2694.83	898.28**	
Intestines (in animal)	4	1349.63	337.41**	
I × D	21	1796.74		
I × Regression	7	799.48	114.21	
A × Regression	3	713.80	256.90**	
I(A) × Regression	4	85.68	21.42	
I × Linearity	14	997.26	71.23	53.64 (d. f. = 46)
Repetition	32	1470.01	45.94	
Total	63	33333.69		

* 5% probability ** 1% probability

Table VI. Deviation from Regression for Intestines

		d. f.	Deviation from Regression	Mean Square
A_1	I_1	2	349.81	174.91**
	I_2	2	393.73	196.87**
A_2	I_1	2	74.41	37.21
	I_2	2	93.84	46.92
A_3	I_1	2	145.65	72.83
	I_2	2	99.40	49.70
A_4	I_1	2	120.56	60.28
	I_2	2	13.06	6.53
		16	1290.46	

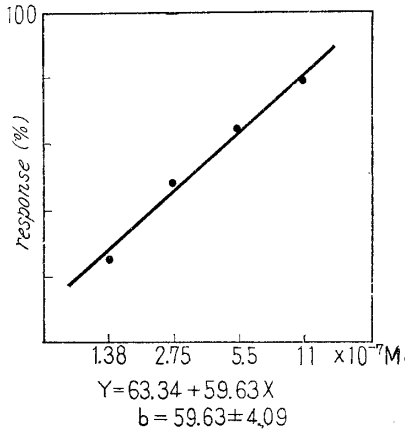

Fig. 4.
Linear Regression of Contractile Responses on log Doses of ACh (Data in Table IV)

(4) Test of linearity on Atr dose-inhibition curve: In the same way as Test (3), observations were made for a test of linearity on Atr dose-inhibition curve. The range of concentrations of Atr was about 3×10^{-9} to 1.2×10^{-7} *M*.

The contraction with 2.2×10^{-6} *M* ACh was inhibited by four dose levels of Atr, i.e. 1.47×10^{-8}, 2.34×10^{-8}, 3.75×10^{-8}, and 6×10^{-8} *M*. The ratios of adjacent doses were made 5:8. The results are shown in Tables VII and VIII, and in Fig. 5.

TABLE VII. Inhibition Ratio at Four Doses of Atropine against 2.2×10^{-6} *M* ACh

	$\times10^{-8}$ *M*	1.47	2.34	3.75	6
A_1	I_1 i	29.4	47.1	73.6	83.8
	I_1 ii	33.8	61.8	75.0	82.4
	I_2 i	44.0	72.7	80.3	84.9
	I_2 ii	45.5	62.2	83.4	90.9
A_2	I_1 i	20.0	36.4	41.9	78.2
	I_1 ii	23.7	47.3	69.1	81.9
	I_2 i	30.0	42.0	53.0	77.0
	I_2 ii	30.0	56.0	69.0	78.0
A_3	I_1 i	23.0	44.2	69.2	78.0
	I_1 ii	29.4	42.7	72.0	81.0
	I_2 i	23.1	53.9	73.1	75.0
	I_2 ii	38.5	50.0	71.2	78.9
A_4	I_1 i	20.9	54.2	73.3	76.7
	I_1 ii	37.5	46.7	71.7	78.3
	I_2 i	29.2	60.4	66.7	87.5
	I_2 ii	25.0	39.6	73.0	92.8
Total		483.6	817.2	1115.5	1305.3
Means		30.2	51.1	69.7	81.6

TABLE VIII. Analysis of Variance for the Data of Table VII

Adjustment for Mean 216341.27

Nature of Variation	d. f.	Sum of Squares	Mean square
Regression	1	23894.78	
Deviation from regression	2	342.27	171.14**
Doses	3	24237.05	
Intestines	7	2043.75	
Animals	3	1539.69	513.23**
Intestines (in animal)	4	504.06	126.02*
I×D	21	696.73	
I×Regression	7	314.11	44.87
A×Regression	3	118.28	39.43
I(A)×Regression	4	195.83	48.96
I×Linearity	14	382.62	27.33 } 40.93
Repetition	32	1500.22	46.88 } (d. f. = 46)
Total	63	28477.75	

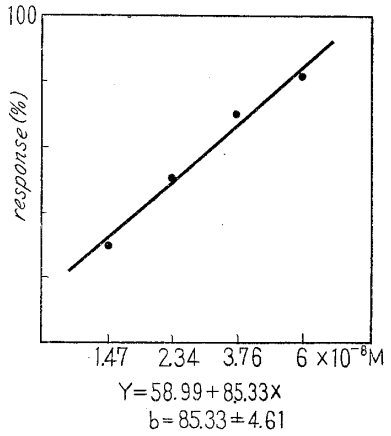

Fig. 5.
Linear Regression of Inhibition Ratio on log Doses of Atropine (Data in Table VII)

Discussion and Conclusion

In this experiment, the isolated intestine of mice used exclusively did not always have better sensitivity to ACh, but there was a good reason to recognize an advantage such as the fact that the variation of response had been less than that with guinea pigs or rabbits and that a great many mice could be had inexpensively. On the other hand, this small animal inevitably had less sensitive response in the cold season.

On optimal temperature in a muscle bath, the results described above definitely indicate that response was affected very little at 20° to 38°. Further, we could not reveal any significant differences of mean values between 38° and other temperatures. It appears, therefore, that optimal temperature of the bath extends over the whole range of 20° to 38°, but it was true that higher temperature made responses more sensitive and increased spontaneous movements of intestinal segments and decrease their fate. From this experiment, temperature of the bath for bioassay may be suitable at about 26°.

On the mode of administration of drugs we find a remarkable difference between (A) and (B). On repeated addition of 1 cc. of $10^{-6}M$ and 0.1 cc. of $10^{-5}M$ ACh, the same intestine showed different response in spite of the same final concentration in the bath. To identify the experimental conditions, therefore, a given volume of each concentration must be administered in a fixed position at a definite period.

On preliminary test for a quantitative analysis, given in Tables V and VIII using a small experiment with four mice, there was no significant difference between "I× linearity" and "repetition" and this shows that these factors are made of σ^2. In the former, "deviation from regression" of mean values of eight observations in each dose was not significant compared with 53.64 which is pooled estimate of two terms, "I× linearity" and "repetition". Consequently, the linearity has been recognized.

To the contrary, not only all factors which are regarded as individual variations are significant but so is "A×regression" in Table V. This fact shows that if this fault is caused by the variation of an animal, better experiment would have to use a larger number of mice than four, but in Table VIII, a similar defect was not indicated.

By further analysis of Table V, "deviation from regression for intestines" has shown by Table VI that there are two significantly deviated values out of eight estimates. These two estimates, which had heretofore been excluded from data by reason of bad animals, must be included in data and considered collectively.

Now, when linearity will be recognised in each block (intestine), test of effective ratio is possible in each block. In such a case, if there is a significant variation among regression coefficients in several blocks, the effective ratio will necessarily vary.

In Table VIII linearity of regression is questioned, but it should not be worried

because, in transforming percentage responses to logit, the observed means scatter about the logistic regression line within a reasonable limit. The variance due to deviation from regression is 78.8 which is far smaller than that in Table VIII. Such a result would be a special case and usually untransformed data also show linearity within responses from 85 to 25% according to our experiences. The significance of logistic transformation and method of calculation will be discussed in the next paper.

We wish to express our thanks to Prof. H. Kumagai and Prof. Y. Ito of University of Tokyo for guidance and help in the course of this work.

Summary

The usefulness and applicability of the small intenstines of mice for the assay of acetylcholine and atropine were examined and the results were treated statistically.

(Received July 13, 1956)

U.D.C. 615.782-015.12

84. **Keijiro Takagi and Masayasu Kimura** : Chemicopharmacological Studies on Antispasmodic Action. VI. On Statistical Treatment of Concentration-Action Relationship of Acetylcholine on Small Intestines of Mice.

(*Pharmaceutical Institute, Medical Faculty, University of Tokyo**)

In order to study antispasmodic action, the mode of acetylcholine (ACh) action requipes to be clarified. This paper is concerned with the relation between ACh and acetylcholine receptor (R), and the theory of Clark[1] on concentration-action curve of ACh has now been recognized by using the statistical method shown in the preceding paper.[2]

Clark offered Langmiur's absorbtion formula to explain the action of ACh by the law of mass-action and then he has shown, though still very far from complete, that this formula could be applied on the concentration-action curve of ACh.

Recently, further observations with isolated guinea pig intestines by Matsumoto[3] supported this Clark's conception, but the tests of goodness of fit seemed to be nothing but graphic.

On the contrary, we have found that the method of logistic transformation was really and truely available for dealing with such observations. The formula (1) derived from the Clark's equation is identical with the formula (2) derived from logistic equation when the β and the K_A are respectively equal to n and $e^{-\alpha}$.

Clark's formula

$$A^n = K_A \frac{y}{y'-y} \longrightarrow e^{n w} = K_A \frac{y}{y'-y} \quad \cdots\cdots(1)$$

Logistic formula

$$P = \frac{1}{1+e^{-(\alpha+\beta X)}} \longrightarrow e^{\beta X} = e^{-\alpha} \frac{y}{H-y} \quad \cdots\cdots(2)$$

$$\longrightarrow \beta X = \log_e \frac{p}{1-p} - \alpha \longrightarrow Y = \beta X + \alpha \quad \cdots\cdots(3)$$

---

* Hongo, Tokyo (高木敬次郎, 木村正康).

1) A. J. Clark : J. Physiol., **61**, 530(1926).
2) K. Takagi, M. Kimura : This Bulletin, **4**, 444(1956).
3) S. Matsumoto : Medical Science, **6**, 113(1954).