101. Keijiro Takagi and Issei Takayanagi : Chemicopharmacological Studies on Antispasmodic Action. IX.[1] Precise Discrimination of Atropine-like Activity and Papaverine-like Activity as Competitive and Non-competitive Antagonism to Acetylcholine.

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We have, up to now, considered that the atropine-like antispasmodics relax the contraction due to acetylcholine (ACh) and that the papaverine-like substances have antibarium action.

The former does not relax the contraction due to barium and on the other hand, the latter also inhibits the contraction due to Ach non-specifically. If a substance had a weaker atropine-like action (A-action) and at the same time a comparatively potent papaverine-like action (P-action), the separate estimation of each activity would become very difficult. Now, if the anti-ACh action could be divided into the competitive and non-competitive antagonistic actions, A-action might come under the former and the P-action under the latter. As the experiments on the competitive anti-ACh action have been previously reported[1,2] we considered generally the non-competitive action, and theoretical and experimental treatment on synthetic antispasmodics, possessing both P- and A-actions, were advanced.

On the non-competitive inhibition, the site of action of antagonist papaverine (P) is different from that of agonist (ACh), so we must assume that P affects some processes beyond the receptor of ACh and there may exist an equilibrium condition between the antagonist (P) and its receptor.

Then the same equation as that in the case of ACh will be considered :

$$e^{mX_p}/K_p = \theta/(1-\theta) \qquad (1\text{-}1)$$

K_p : Dissociation constant between P and its receptor

θ : Ratio of receptors occupied by the inhibitor (P)

m : No. of inhibitor P-molecules which block one contractive unit

The response to a definite concentration of ACh in the presence of various concentrations of inhibitor (P) is in proportion to $(1-\theta)$. When X_p is very small, viz. $X_p \to 0$, the response to X_A is y'' and the response y in the presence of both X_A and X_p may be given as follows :

$$y = y''(1-\theta) \qquad (1\text{-}2)$$

From (1-1) and (1-2)

$$e^{mX_p}/K_p = (y''-y)/y \qquad (2\text{-}1)$$

$$y'' = \frac{e^{X_A}/K_A}{1+e^{X_A}/K_A} \, y' \qquad (2\text{-}2)$$

From (2-1) and (2-2)

$$e^{X_A}/K_A = y/(y'_p - y) \qquad (3\text{-}1)$$

$$y'_p = y'/(1+e^{mX_p}/K_p) \qquad (3\text{-}2)$$

Here, y'_p is the maximum response at X_p (see Fig. 2).

The equations (2-1) and (2-2) show the log dose-inhibition curve of the inhibitor (P) (Fig. 1) and the equations (3-1) and (3-2) the log dose-contraction curve of agonist X_A in the presence of X_p (Fig. 2).

As the usual synthetic antispasmodics (B) have A- and P-actions, two different

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* Hongo, Tokyo (高木敬次郎, 高柳一成).

1) Part Ⅷ. K. Takagi, M. Kimura : This Bulletin, **5**, 440(1957).

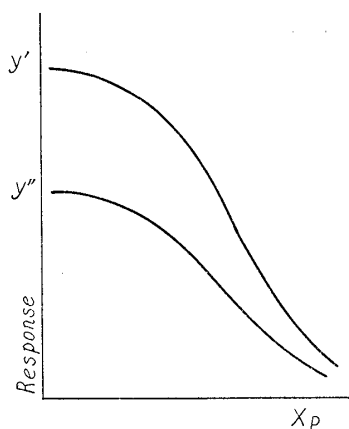2) K. Takagi, I. Takayanagi : *Ibid.*, **5**, 247(1957).

Fig. 1. Schematic Inhibition Curves by
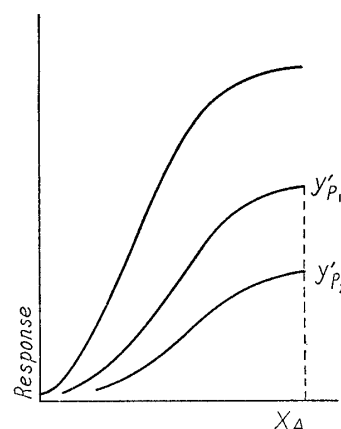Non-competitive Antagonist (P)

Fig. 2. Schematic Contraction Curves in the
Presence of Non-competitive Antagonist (P)

factors must be considered at the same time; that is, a parallel shift of ACh dose-contraction curve by A-action and an inhibition of the maximum contraction by P-action.

Then the equation (4-1) accounts for A-action of B and (4-2) is given for P-action.

$$\frac{e^{X_A}}{K_A\left(1+\dfrac{e^{nX_B}}{K_B}\right)}=\frac{y}{y'_B-y} \tag{4-1}$$

$$y'_B=y'\frac{1}{1+\dfrac{e^{mX_B}}{K'_B}} \tag{4-2}$$

K_B : Dissociation constant between B and ACh-receptor.

K'_B : Dissociation constant between B and papaverine-receptor.

y'_B : Maximum contraction by ACh in the presence of X_B.
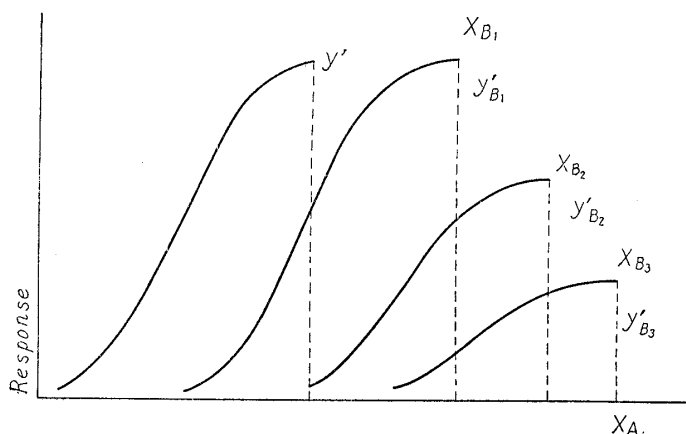
These epuations can be shown in Fig. 3.

Fig. 3.

Schematic Contraction Curves in the
Presence of Competitive and Non-competitive Antagonist (B)

From the equation (4) will be given equations (5-1) and (5-2) :

$$\frac{e^{nX_B}}{K_B\left(1+\dfrac{e^{X_A}}{K_A}\right)}=\frac{y''_B-y}{y} \tag{5-1}$$

$$y''_B=y'_B\frac{e^{X_A}/K_A}{1+e^{X_A}/K_A} \tag{5-2}$$

Then from (2-2), (4-2), (5-1), and (5-2), equation (6-1) is given :

$$\frac{e^{nX_B}}{K_B\left(1+\dfrac{e^{X_A}}{K_A}\right)} + \frac{e^{mX_B}}{K'_B} + \frac{e^{nX_B}}{K_B\left(1+\dfrac{e^{X_A}}{K_A}\right)} \cdot \frac{e^{mX_B}}{K'_B} = \frac{y''-y}{y} \qquad (6)$$

This equation (6) suggests the inhibition curve having both actions (cf. dotted line $A \times P$ in Fig. 4).
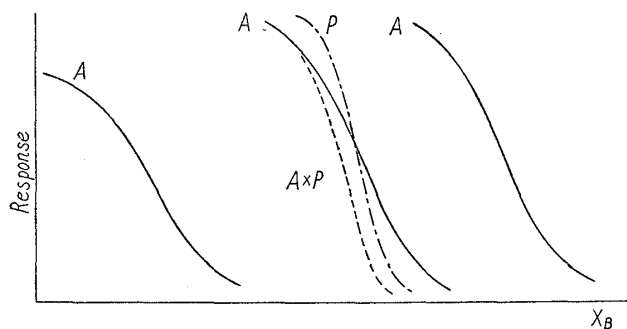

Fig. 4.

Schematic Representation of Inhibition Curves in the Presence of Competitive and Non-competitive Antagonist (B)

A : Atropine-like inhibition curves of B.
P : Papaverine-like inhibition curve of B.
A × P : Combined inhibition curve of B.

In assaying such antispasmodics, we must consider two inhibitory action separately.

The effective concentration of the papaverine-like activity that is represented by the equations (2-1) and (2-2) might be constant irrespective of the concentration of ACh, but the fact that the effective concentration of the atropine-like activity is influenced by the concentration of ACh was shown previously by one of us.[1] The two kinds of antagonistic actions which can be traced as in Fig. 4 are so characteristic that they may be separated completely, if suitable concentration of ACh be selected. Of course, it must be assumed that K_B is smaller than K'_B as found in the usual antispasmodics.

In the equation (6), when X_A and X_B are lowered simultaneously, the 2nd and 3rd items become smaller than the 1st item, for K_B is smaller than K'_B and then it agrees with the equation for A-action.

To the contrary, when e^{X_A} becomes extremely higher, the equation (2-1) that represents a papaverine-like inhibition curve was formed, because the 1st and 3rd items can be neglected, but if K_B is not extremely small compared to K'_B, such an omission would be incorrect.

Methods

Healthy male mice, weighing 15~20 g., were used. The method of making isolated ileum preparation and recording contractile response was the same as in the previous reports.[1,3]

After the ileum was bathed in the Tyrode solution containing the antagonist, the solution containing various concentrations of ACh was injected into the fluid and maximum contraction was measured.

The fluid was then replaced with a Tyrode solution and the preparation was washed 2~3 times over a period of at least 8 mins.

For obtaining the log dose-response curves of the P- and A-actions, the following experimental design was applied after the sensitivity to a definite concentration of ACh was established to a similar level.

Experimental Design

The experimental designs were the same as those described in our previous paper,[2] and for obtaining the potency ratios of each of the two actions of the antispasmodics used, two doses were alloted to each drug and the order of the drugs was chosen at random on the same intestines.

The graphic method described in the previous papars[4,5] was used to treat the experimental data.

3) K. Takagi, M. Kimura : This Bulletin, 4, 444(1956).
4) K. Takagi, et al. : J. Pharm. Soc. Japan, 76, 1187(1956).
5) K. Takagi, et al. : Ibid., 76, 1196(1956).

Experimental Results

I] **Dose-Response Relationship of Avacan and Papaverine**—The response to the two compounds was followed experimentally in detail. Investigating the contraction curves it was shown that both compounds have competitive and non-competitive actions, especially that A-action of papaverine, which had been reported by Chihara,[6] is found only after a logistic transformation of the original data (Figs. 5 and 6, and Table I).
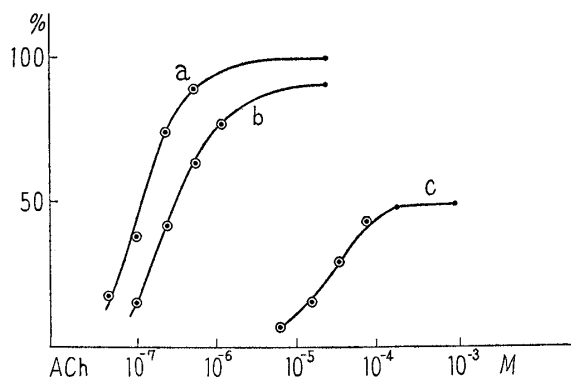
Fig. 5.

Movement of ACh Dose-Response
Curves by Avacan

Curve a, ACh alone
Curve b, with 8×10^{-7} g./cc. of Avacan
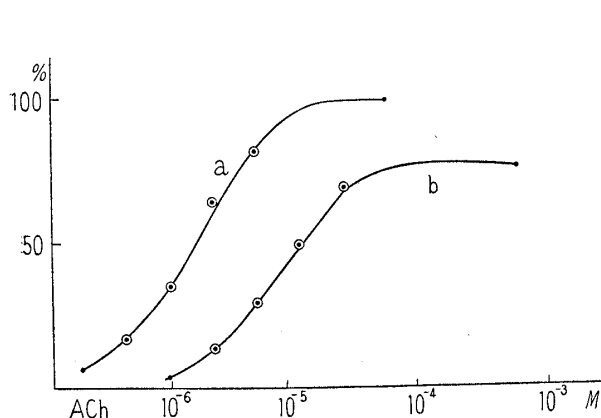Curve c, with 4×10^{-6} g./cc. of Avacan

Fig. 6a. Movement of ACh Dose-Response
Curves by Papaverine

Curve a, ACh alone
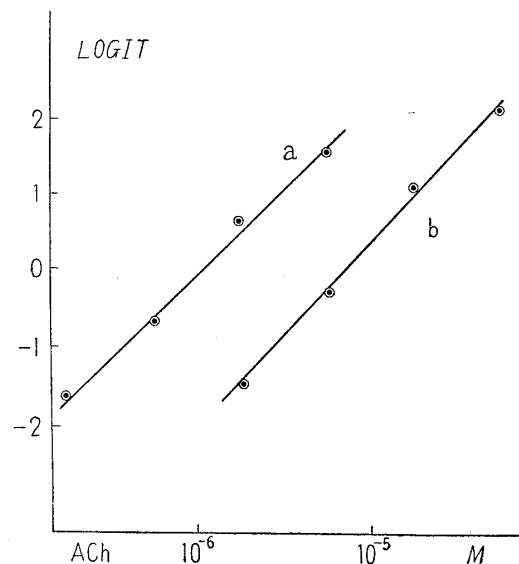Curve b, with 2×10^{-6} g./cc. of papaverine

Fig. 6b. Logistic Curves of Fig. 6a

TABLE I. Movement of ACh Dose-Response Curves by Antagonists

Nature of variation		Avacan		Papaverine	
		d.f.	Mean square	d.f.	Mean square
Deviation from regression	a)	2	0.0542	2	0.0116
	b)	2	0.0621	2	0.0117
	c)	2	0.0655		
Parallelism		2	0.0076	1	0.0070
Between doses†		11	0.0875*	7	0.0935*
Between animals		11	0.7806**	9	0.5824**
Error		121	0.0246	63	0.0158
Total		143		79	

a) b=0.906, $\beta=1$, $t_0=0.9311$††   a) b=0.946, $\beta=1$, $t_0=1.792$††
b) b=0.964, $\beta=1$, $t_0=0.0181$ b) b=0.991, $\beta=1$, $t_0=0.0889$
c) b=0.863, $\beta=1$, $t_0=0.6230$

In all the Tables in this report, the following notations are used.
† ⊙ in the corresponding figures was used for this calculation.
†† $t_0=(b-\beta)/\sqrt{V(b)}$.
††† $p=0.05$.

6) R. Chihara : Fol. Pharmacol. Japon., **52**, 141(1956).

Two kinds of inhibition curve were obtained with the same intestines, each with Avacan and papaverine (Figs. 7 and 8, and Table II).
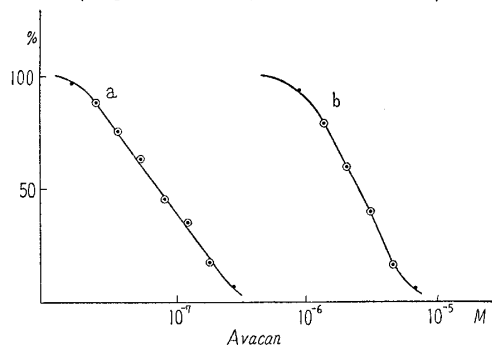

Fig. 7a. Dose-Inhibition Curves of Avacan

Curve a=Atropine-like inhibition to
4×10⁻⁸ g./cc. of ACh
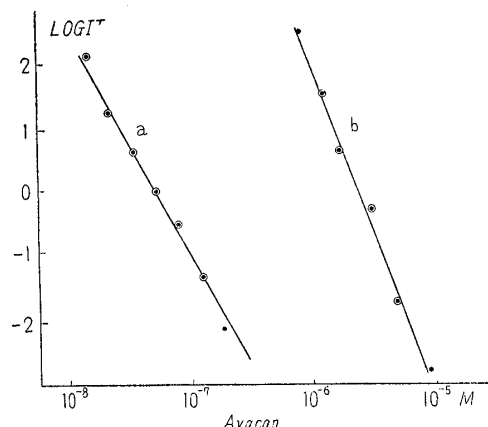Curve b=Papaverine-like inhibition to
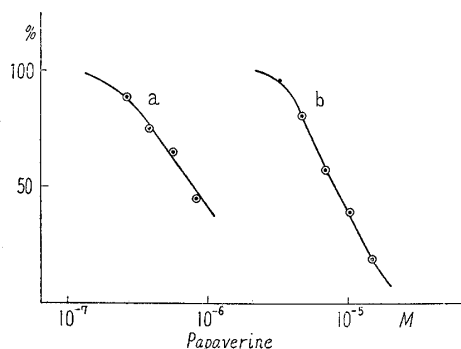1.11×10⁻⁴ g./cc. of ACh

Fig. 7b. Logistic Curves of Fig. 7a

Fig. 8

Dose-Inhibition Curve of Papaverine

Curve a=Atropine-like inhibition to
4×10⁻⁸ g./cc. of ACh
Curve b=Papaverine-like inhibition to
1.11×10⁻⁴ g./cc. of ACh

Table II. Dose-Inhibition Curves of Antagonists

Nature of variation		Avacan		Papaverine	
		d.f.	Mean square	d.f.	Mean square
Deviation from regression	a)	4	0.0271	2	0.0167
	b)	2	0.0058	2	0.0214
Between doses†		9	0.0336*	7	0.1168*
Between animals		11	0.6222**	9	0.4239**
Error		99	0.0767	63	0.0105
Total		119		79	

a) $b=2.275$ a) $b=2.110$
b) $b=1.405$, $\beta=1.5$, $t_0=0.3318$††   b) $b=1.517$, $\beta=1.5$, $t_0=0.072$††

The reaction orders of the A-action of the two antagonists examined here were almost the same with that of atropine ($n=1.5$) and those of the P-action were similar to each other ($m=2.0\sim 2.5$). The slope of the non-competitive inhibition curve was so large, that is, the range of the effective concentration of P-action was so narrow, that even in papaverine the competitive inhibition curve could be separated from the non-competitive one by selecting an appropriate concentration of ACh.

II] Potency Ratios of Some Antispasmodics—Here were used 6 antispasmodics, of which the regression lines were recognized as parallel, both in A-action and in P-action (Fig. 9 and Table III).

Table III. Assay of P- and A-Actions of Six Antispasmodics

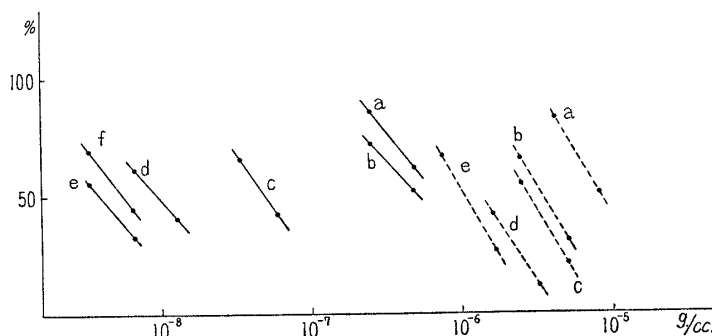| Nature of variation	P-Action		A-Action	
	d.f.	Mean square	d.f.	Mean square
Parallelism	4	0.0531	5	0.0360
Between doses†	9	0.2306*	11	0.3397*
Between animals	13	0.6332**	9	0.4263**
Error	117	0.0272	99	0.0288
Total	139		119	

bc=2.36   bc=1.469

Fig. 9.

Solid line = Atropine-like action to 4×10^{-8} g./cc. of ACh
Dotted line = Papaverine-like action to 1.11×10^{-4} g./cc. of ACh
a) = Dihydroneuspasverine (1-Piperonyl-3-methyl-6,7-methylendioxy-3,4-dihydroisoquinoline)
b) = Papaverine hydrochloride
c) = Avacan (Isoamyl β-dimethylaminoethylaminophenylacetate hydrochloride)
d) = Aspaminol (1,1-Diphenyl-3-piperidinobutanol hydrochloride)
e) = Benactyzine (Diethylaminoethyl benzilate hydrochloride)
f) = Atropine sulfate

Non-competitive activity ratio as the P-action to the high concentration of ACh (1.11×10^{-4} g./cc.) and the competitive activity ratio as the A-action to the low concentration of ACh (4×10^{-8} g./cc.) are given in Table IV.

Table IV. Potency Ratio of Six Antispasmodics

	P-Action		A-Action	
	Ratio	Fiducial limit[†††]	Ratio	Fiducial limit[†††]
Atropine	0(0)		100(100)	
Avacan	117(100)	209~ 64	11(15)	16.2 ~ 6.3
Aspaminol	235(50)	297~152	59(47)	79.5 ~48.5
Benactyzine	320(150)	395~213	140(120)	198 ~81
Papaverine	100(100)		1.2(−)	2.52~ 0.72
Dihydroneuspasverine	24(−)	39~ 18	0.8(−)	1.07~ 0.52

Figures in parentheses indicate papaverine-like activity ratios as antibarium action or the atropine-like activity ratios as anti-ACh action tested with the ileum of guinea pigs in the previous report.[7]

Further, the antibarium action and the non-competitive anti-ACh action of three compounds were compared with intestines of mice and also of guinea pigs (Table V).

TABLE V. Antibarium and Non-competitive Antiacetylcholine Action in Intestines of Mice and Guinea Pigs

	Against BaCl$_2$ (3.33×10^{-4} g./cc.)		Against ACh (1.11×10^{-4} g./cc.)	
	Mice	Guinea pigs	Mice	Guinea pigs
Papaverine	100	100	100	100
Aspaminol	148	214	171	247
Dihydroneuspasverine	25	40	29	49

From the above results, the potency ratio in relaxing the contraction due to the high concentration of ACh was almost the same as the ratio against BaCl$_2$ in each animal species.

III] Action of Nicotine—3.33×10^{-5} g./cc. nicotine contracted 11 of 36 isolated ileums of mice and $10^{-6} \sim 10^{-7}$ g./cc. of atropine relaxed these contracted ileums. At a larger dose than 3.33×10^{-5} g./cc. of nicotine, however, a number of ileums was relaxed, so the dose-response curve of nicotine and the relationship between atropine and nicotine was not established fully.

Discussion

Because the action of barium upon intestine is at least partly mediated by stimu-

7) K. Takagi, Y. Kasuya, Y. Ota : J. Pharm. Soc. Japan, 73, 541(1953).

lation of ganglion cells,[8] antibarium activity of some compounds differ considerably by the nervous structures of gut.

By the above reason, Parkes stated barium to be inappropriate as the spasmogens for the assay of P-action.[9] We have used now high concentration of ACh for assaying P-action. ACh may also be ganglionic stimulant in addition to the neuro-effector action and may not be free from the same defect as barium. However, the action of high concentration of ACh is far more unequivocal than that of barium and the distinction of the P-action from A-action, which will be detected by low concentration of ACh, is not very difficult.

The fact that the maximum contraction by an extremely high concentration of ACh could not be depressed with the concentration of atropine which in turn was able to inhibit the nicotine contraction of mouse intestine, might indicate the depression of the maximum contraction by some antispasmodics to be non-competitive and not to be ganglionic in nature.

The slope of ACh-contraction curve was not influenced by Avacan, papaverine, or atropine. All the results obtained in our laboratory indicate that the reaction order of ACh to its receptor is not significantly deviated from 1, but because the results from other laboratories do not always coincide with the theoretical value of 1, Stephenson[10] tried to modify the Clark's original theory due to the law of mass action.[11] The slope of A-action is also different from the value expected theoretically, although all the compounds used have the common slope (n=1.5). In spite of such uncertainties in the slopes of action curves, there will be no reason to doubt the relative shift of the curves by competitive antagonists.

Many authors[1,12~14] have recognized this phenomenon of mutual antagonism and this is utilized by us to differentiate between A- and P-actions. A full examination of the contraction curves under various concentrations of the antagonists might reveal its inhibitory potencies, but this method is too complicated for use as a routine assay. Our new method, comparing two kinds of inhibition curves, is far simpler and can compare several drugs at once.

The non-competitive anti-ACh activities of the antispasmodics used agree fairly well with the antibarium activities which had been obtained in our previous paper[7] and which were newly observed in intestines of mice and guinea pigs (Table V). As the submaximum concentration of ACh $(10^{-6} g./cc.)$ is used as spasmogens in a customary method[15] for estimating neurotropic activity, it is necessary to use a correspondingly higher concentration of antispasmodics, which may become closer to the concentration exerting antibarium action. This will cause the overlook of a weak anti-ACh action in papaverine.

An essential nature of antispasmodic actions will be made clearer when the precise activity of competitive and non-competitive anti-ACh action of many compounds having different chemical structures has been estimated.

8)   W. Feldberg : J. Physiol., **113**, 483(1951).
9)   M. W. Parkes : Brit. J. Pharmacol., **10**, 95(1955).
10) R. P. Stephenson : *Ibid.*, **11**, 379(1956).
11) A. J. Clark : J. Physiol., **61**, 530(1926).
12) P. B. Marshall : Brit. J. Pharmacol., **10**, 354(1955).
13) A. R. Timms : *Ibid.*, **11**, 273(1956).
14) H. Matumoto : Medical science, **6**, 113(1954).
15) L. C. Miller, *et al.* : J. Pharmacol. Exptl. Therap., **92**, 260(1948).

Summary

(1) ACh dose–response curve in the presence of various concentrations of antagonist was traced fully and it was suggested experimentaly that the curve moved parallel by the atropine–like action and its maximum response was depressed non-competitively by the papaverine–like action.

(2) It was made possible to discriminate precisely the atropine–like action as competitive antagonism to a low concentration of ACh 4×10^{-8} g./cc. and the papaverine-like action as non–competitive antagonism to a high concentration of ACh $(1.11 \times 10^{-4}$ g./cc.).

(3) The potency ratios in relaxing the contraction due to the high concentration of ACh was almost the same as the ratios against $BaCl_2$.

102. Tsukasa Kuraishi : 4,5–Substituted Pyridazines. III.[1] Oxidation and Solvolysis of 4–Methyl–3,6–dichloropyridazine.

(*Pharmaceutical Faculty, University of Nagasaki**)

In connection with the synthesis of 4,5–substituted pyridazines, which was reported in the previous papers, the oxidation and solvolysis of 4–methyl–3,6–dichloropyridazine (I) were studied and this paper describes the oxidation of the methyl group in the 4–position of some pyridazine compounds.

Very recently, Takabayashi[2] reported the synthsis of 4–methyl–6–chloro–3–pyridazinol (IIIa) and 5–methyl–6–chloro–3–pyridazinol (IIIb) by heating (I) with sodium hydroxide solution, using hydrous and 50% methanol solution, and the isomers were separated by fractional recrystallization from methanol or ethanol.

Previously, the writer[1] carried out the solvolysis of 3,6–dichloro–, 3,4,6–trichloro–, and 3,4,5–trichloropyridazines with glacial acetic acid and in the present paper, reports the result of the same experiment (I) performed by the same method.

By oxidation with excess of potassium dichromate in conc. sulfuric acid, (I) was easily converted to 4–carboxy–6–chloro–3–pyridazinol (II), which recrystallized from water as a monohydrate.

Catalytic reduction of (II) with palladium–charcoal yielded 4–carboxy–3–pyridazinol (IV) which was identical with the sample derived from 4–cyano–3–pyridazinol (V) by Druey's method.[3]

On the other hand, when (I) was refluxed with glacial acetic acid for one hour, (IIIa) and (IIIb) were formed and were separated by pouring the mixture into five volumes of water. (IIIb) deposited on standing at room temperature and (IIIa) was obtained from the filtrate by evaporation to dryness *in vacuo*. Each of these products was purified by recrystallization from water.

Oxidation of (IIIa) and (IIIb) by similar procedure respectively gave in poor yields

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  * Schowa–machi, Nagasaki (倉石　典).
1) Part II : This Bulletin, **5**, 376(1957).
2) N. Takabayashi : *Ibid.*, **5**, 229(1957).
3) P. Schmidt, J. Druey : Helv. Chim. Acta, **37**, 134(1954).