UDC 615.784.2-015.25

77. Keijiro Takagi and Masayasu Kimura : Chemicopharmacological Studies on Antispasmodic Action. VIII. On the Antagonistic Action of Atropine and its Methobromide to Acetylcholine with the Isolated Small Intestines of Mice.

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

The physiology and pharmacology of small intestines were reviewed by Williams[1] and the pharmacological action of atropine (Atr) upon neuro-effector transmission was considered in the review by Ambache.[2]

In view of the complex mechanism involved in physiological contraction of smooth muscles, it is astonishing that such a simple equation as that derived by Clark[3] fits reasonably well the concentration-action relationship of the agonists.[4] Now, our attention was directed to the antagonistic action of Atr to acetylcholine (ACh), which was considered as mutually competitive. As we became aware, in advancing the experiment, that Atr sensitized the contractility of excised small intestines after its wash-out, we investigated at first the effect of Atr on the sensitivity and contractility of isolated small intestines. Finally, the action of Atr was compared with that of atropine methobromide (Atr-MeBr) which is a quaternary ammonium salt of Atr.

Experimental Method

Biological condition and experimental method were the same as those described earlier,[5] using excised ileums of mice, which were immersed in Tyrode solution containing Atr for 5 mins. before administration of ACh. After washing out the Atr solution, a resting period of 10 mins. was inserted before the next application of drugs. The order of application of drugs in the same preparation was randomized in all cases. Experimental designs were as follows :

I. Effect of Atr on the Reaction of Small Intestines to ACh

(i) Two kinds of treatment, one with ACh alone (9.9×10^{-8}, 2.48×10^{-7}, 6.05×10^{-7} *M*) and the other with Atr (1.16×10^{-6} *M*) and ACh (6.05×10^{-7}, 1.54×10^{-6}, 3.8×10^{-6} *M*), were applied in the same preparations. Ten animals used were divided into 2 groups. (a) One group was treated at first with ACh, then, after 10 mins., with Atr and ACh, and (b) the other group in the reversed order.

(ii) In the same intestines (a) 3 doses of ACh (1.38×10^{-7}, 3.47×10^{-7}, 8.8×10^{-7} *M*) and (b) after 10 mins., ACh (8.8×10^{-7}, 2.2×10^{-6}, 5.5×10^{-6} *M*) with Atr (1.16×10^{-8} *M*) were applied. The intestines were washed several times and allowed to rest for 1 hr. Then (c) another 3 doses of ACh (2.2×10^{-8}, 5.5×10^{-8}, 1.38×10^{-7} *M*) were added. In each stage of the experiments the maximum contraction was produced by higher concentration of ACh.

(iii) Responses to 3 doses of ACh (5.5×10^{-8}, 1.38×10^{-7}, $3.47\times10\,M^{-7}$) were observed 1 hr. after the first application of ACh (1.38×10^{-7}, 3.47×10^{-7}, 8.8×10^{-7} *M*) on the same intestines.

II. Split-plot Design for Testing the Antagonistic Action of Atr to ACh[6]

Six ACh doses were applied under a definite concentration of Atr in the same preparation and 6 Atr doses were assigned to 6 preparations, which were made to form one replication. The concentration of ACh and Atr used in this design are indicated in Table V. A total of 5 replications were carried out, consisting of 30 animals.

III. Concentration-Inhibition Relationship of Atr

The contraction elicited by ACh (2.2×10^{-6} *M*) was inhibited by a series of Atr doses in the same preparations, using 5 animals.

* Hongo, Tokyo (高木敬次郎, 木村正康).

1) E.M.V. Williams : Pharmacol. Rev., **6**, 159(1954).
2) N. Ambache : *Ibid.*, **7**, 467(1955).
3) A.J. Clark : J. Physiol., **61**, 530(1926).
4) K. Takagi, M. Kimura : This Bulletin, **4**, 449(1956).
5) K. Takagi, M. Kimura : *Ibid.*, **4**, 444(1956).
6) W.G. Cochran, G.M. Cox : "Experimental Designs," 218(1950).

TABLE I. Periodical Change of Effect after Atropine Wash-out

			Experimental design: Repetition	Experimental design: Conc. of ACh (M)	Mean responses (%): Before Atr	Mean responses (%): By Atr	Mean responses (%): After 10′	Mean responses (%): After 60′
After-effect of Atr	i	a	4	9.90×10^{-8}	20.4			
			4	2.48×10^{-7}	45.4			
			4	6.05×10^{-7}	68.8	16.3		
			4	1.54×10^{-6}		44.5		
			4	3.80×10^{-6}		78.9		
		b	6	9.90×10^{-8}			22.0	
			6	2.48×10^{-7}			50.7	
			6	6.05×10^{-7}		20.4	77.5	
			6	1.54×10^{-6}		44.0		
			6	3.80×10^{-6}		77.7		(c)
	ii		5	2.20×10^{-8}				29.3
			5	5.50×10^{-8}	(a)			53.3
			5	1.38×10^{-7}	34.6			77.7
			5	3.47×10^{-7}	67.7	(b)		
			5	8.80×10^{-7}	86.2	36.9		
			5	2.20×10^{-6}		66.7		
			5	5.50×10^{-6}		87.1		
Without Atr	iii		6	5.50×10^{-8}				44.6
			6	1.38×10^{-7}	55.6			68.6
			6	3.47×10^{-7}	74.4			79.3
			6	8.80×10^{-7}	90.9			

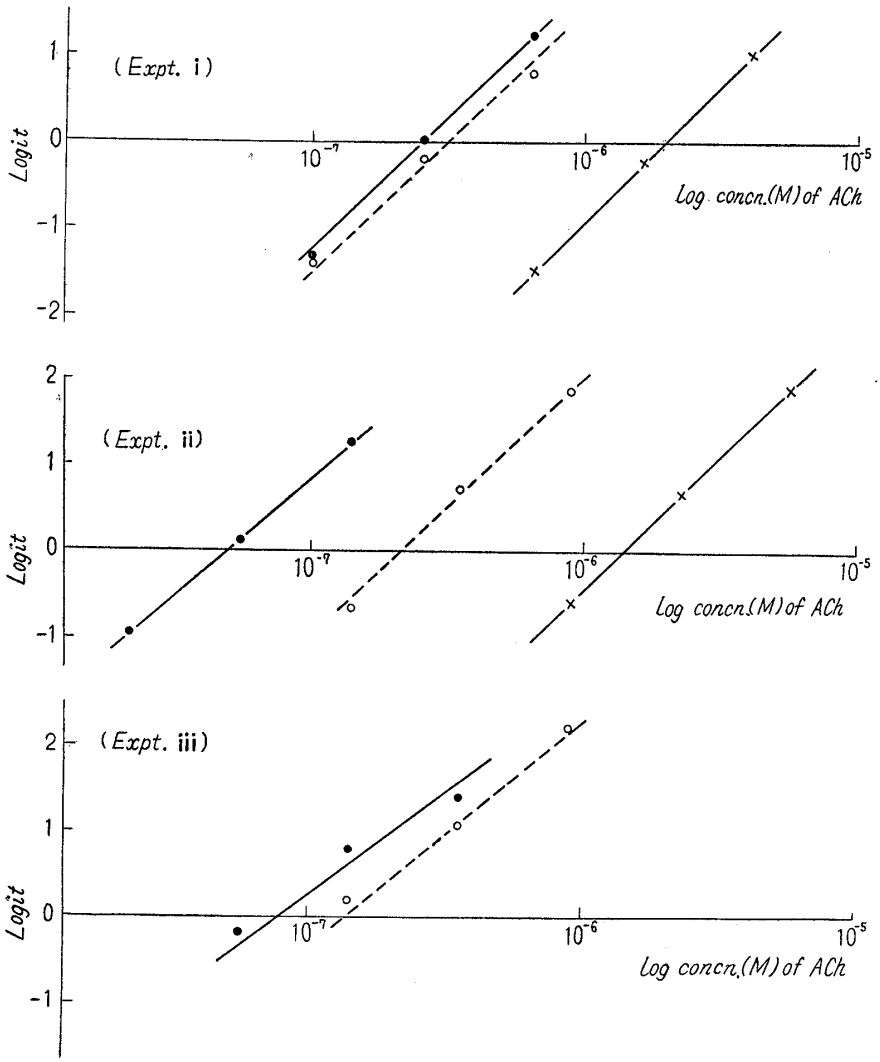

Fig. 1.
Periodical Change of ACh
Logit Lines in Table I

○ : Response on ACh before Atr
● : Response on ACh after Atr wash-out
× : Response on ACh by Atr

IV. Concentration-Inhibition Relationship of Atr-MeBr

The concentration-inhibition curve of Atr-MeBr was traced in the same design as the Experiment III with 5 animals. The sample used was acetylcholine chloride (Hoffmann-La Roche), atropine sulfate (Merck), and atropine methobromide which was generously supplied by the Takeda Pharm. Ind., Ltd.

Results

I. Effects of Atr on the Reaction of Mouse Small Intestines to ACh

Three experiments were completed with the results shown in Table I and Fig. 1. A higher concentration of ACh ($5.5\times10^{-5}\,M$) was necessary to make the response maximum in the presence of Atr.

Investigating the periodical change of effect of ACh after wash-out of Atr, comparison between the effects after 10 min. and 1 hr. can be instituted by Expt. (i) and (ii).

Changes in the effect of ACh in the absence of Atr can be noticed by Expt. (iii). These results are summarized for ready comparison in Table II. In order to test for parallel shift of ACh concentration-action curve by Atr, the data which consist of 6 means obtained from Expt. (i), a and b are represented in Table III.

Table II. Comparison of Sensitivity of Intestine to ACh in Table I

Time	ACh alone	Effect after Atr		Effect without Atr
		10′	60′	
Sensitivity ratio	1	1.26	6.37	1.92

The specificity of Atr sensitization was definitely established, because the sensitizing effect of ACh after 1 hr. of the first medication was shown to be relatively slight compared to that of Atr.

Such effect of Atr was dependent on a resting period after atropinization and the comparatively shorter period of 10 mins. between succeeding experiments, used in this report, would exert little influence upon the sensitivity of intestines.

Table III. Data of Two Concentration-Action Curve of ACh in Presence and Absence of Atr

ACh (M)	Atr (M)	Repetition	Means (%)
9.90×10^{-8}	—	10	21.9
2.48×10^{-7}	—	10	49.2
6.05×10^{-7}	—	10	75.0
6.05×10^{-7}	1.16×10^{-8}	10	19.7
1.54×10^{-6}	1.16×10^{-8}	10	42.6
3.80×10^{-6}	1.16×10^{-8}	10	75.7

Table IV gives the results of test of parallelism for two logistic regression lines obtained from concentration-action curves of ACh alone and of ACh by Atr ($1.16\times10^{-8}\,M$) in Table III, where it is found that the mean square for parallelism 45.7 ($d.f.=1$) is smaller than that for error 57.50 ($d.f.=45$). As parallelism was recognized, it was therefore shown that Atr was not able to change the slope of concentration-action relationship of ACh. In addition to this result, it was found that the maximum response of ACh was not depressed by Atr.

Table IV. Test of Parallelism for Two Logistic Regression Lines*

Nature of Variance	$d.f.$	Mean Square
Deviation from the combined line	3	40.43
Deviation from the individual lines	2	42.80
Parallelism	1	45.70
Error (Doses × Animals)	45	57.50

* cf. Snedecor: "Statistical Method," p. 327.

II. Antagonistic Action of Atr to ACh according to Split-plot Design

The results of the split-plot design are given in Tables V and VI, and in Fig. 2.

In this design the comparison between sub-units, which are performed in the same animal, can be estimated with the error variation within animals (error (II) in Table VI), so that the deviations from theoretical logistic regression lines and parallelism of regression lines may be more precisely estimated than Atr effect, which on the contrary, must be compared with the variation between animals (error (I) in Table VI).

Table VII shows regression equations of 6 ACh action curves and these mean squares of linearity D_0^2 are all smaller than error (II). By this, these equations showed no indication of serious deviation from the experimental data.

TABLE V. Data of Concentration-Action Relationship of ACh at Six Concentrations of Atropine due to Split-plot Design
(Each data represent means of 5 animals)

Atr (M) \ ACh (M)	6.88×10^{-8}	1.38×10^{-7}	2.75×10^{-7}	5.50×10^{-7}	1.10×10^{-6}	2.20×10^{-6}	4.40×10^{-6}	8.80×10^{-6}	1.76×10^{-5}	3.56×10^{-5}	7.16×10^{-5}
2.88×10^{-9}	10.9	17.5	31.3	64.0	76.0	85.3					
5.76×10^{-9}		8.6	18.3	43.3	59.8	76.0	84.4				
1.16×10^{-8}			9.6	20.2	33.7	55.0	73.4	86.2			
2.32×10^{-8}				8.0	13.9	31.8	57.4	79.7	86.5		
4.64×10^{-8}					7.3	17.9	38.4	62.3	79.5	90.6	
9.28×10^{-8}						8.3	16.4	33.4	51.8	75.7	88.0

TABLE VI. Analysis of Variance for Table V

Adjustment for means		425677.84	
Nature of Variance	$d.f.$	Sum of squares	Mean square
Animals (Main Unit)	29	5050.57	174.15
Repeat	4	1096.63	274.16
Atropine	5	315.78	63.16
Error (I)	20	3638.16	181.91
Acetylcholine (Sub Unit)	30	163090.76	5436.36**
ACh (Combined)	5	161546.33	32309.27**
ACh (Combined) × Atr	25	1544.43	61.78
Error (II)	120	8362.90	69.69
Total	179	176504.23	

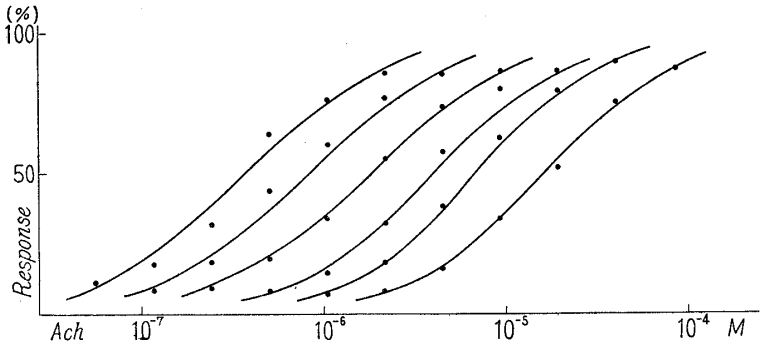

Fig. 2.
Concentration-Action Curves of ACh Antagonized by Atr

TABLE VII. Regression Equations of ACh-Action Curves and Test of Parallelism for 6 Logistic Regression Lines

$$\left.\begin{aligned} Y_1&=1.16\,X+17.07\\ Y_2&=1.15\,X+16.15\\ Y_3&=1.15\,X+15.30\\ Y_4&=1.29\,X+16.20\\ Y_5&=1.36\,X+16.22\\ Y_6&=1.29\,X+15.71 \end{aligned}\right\} \quad Y_c=1.22\,X+15.71$$

Variation	$d.f.$	Mean Square
Deviation from the combined line	29	59.84
Deviation from the individual lines	24	60.12
Parallelism	5	58.47
Error (II)	120	69.69

In Table VII, moreover, the mean square for parallelism 58.47 ($d.f.=5$) is smaller than the error mean square 69.69 ($d.f.=120$) in Table VI so that parallelism of 6 lines may be recognized,

III. Concentration-Inhibition Relationship of Atr to Contraction by ACh ($2.2\times10^{-6}M$)

In Table VIII is shown a comparison between the results in the split-plot design and in the same preparation.

Mean squares for deviation from regression lines in these cases are tabulated in Table X. In the split-plot design, D_0^2 ($=32$) must be compared with some value between error (I) and error (II) in Table VI, but the fact that D_0^2 is smaller than the smaller value ($=69.7$) of the two errors strongly suggests that the concentration-inhibition relationship of Atr obeys the law of mass action.

Table VIII. Comparsion of Data for Concentration-Inhibition Curves of Atr by Different Design of Assay

Atr (M)	By Split-plot		By randomized block design in the same preparation	
	N	%	N	%
2.88×10^{-9}	5	91.7	6	89.8
5.76×10^{-9}	5	82.3	6	72.8
1.16×10^{-8}	5	59.1	6	55.8
2.32×10^{-8}	5	34.2	6	29.6
4.64×10^{-8}	5	19.2	6	14.2
9.28×10^{-8}	5	8.9	6	4.7

IV. Concentration-Inhibition Relationship of Atr-MeBr

The results are shown in Table IX and it was found that Atr-MeBr (1.04×10^{-8} M) cannot inhibit maximum contraction by higher ACh doses (5.5×10^{-5} M). In Table X and Fig. 3 are collected the equations of the regression lines made in Expt. III and IV. From Table X it was shown by t-test that the slope of Atr does not deviate significantly from the supposed value of 1.5 and yet that of its methobromide becomes greater.

Table IX. Data of Concentration-Inhibition Curve of Atropine Methobromide

Atr-MeBr (M)	N	%
2.6×10^{-9}	5	91.9
5.2×10^{-9}	5	81.3
1.04×10^{-8}	5	49.0
2.08×10^{-8}	5	19.9
4.16×10^{-8}	5	10.3

Table X. Comparsion of the Regression Lines in Fig. 3

Sample	By split-plot	By the randomized block method	
	Atropine	Atropine	Atr-MeBr
Regression line	$Y=-1.366\,X+24$	$Y=-1.577\,X+28$	$Y=-1.882\,X+34.5$
Mean square for linearity	$D_0^2=32$	$D_0^2=27$	$D_0^2=39$
$\sqrt{V(b)}$	0.081	0.077	0.135
Supposed value of b	1.5	1.5	1.5 2.0
t-Test	$t_0=1.65$	$t_0=1.00$	$t_0=2.829$ $t_0=0.828$
	$t=2.78$ ($d.f.=4$, $p=0.05$)		$t=3.182$ ($d.f.=3$, $p=0.05$)

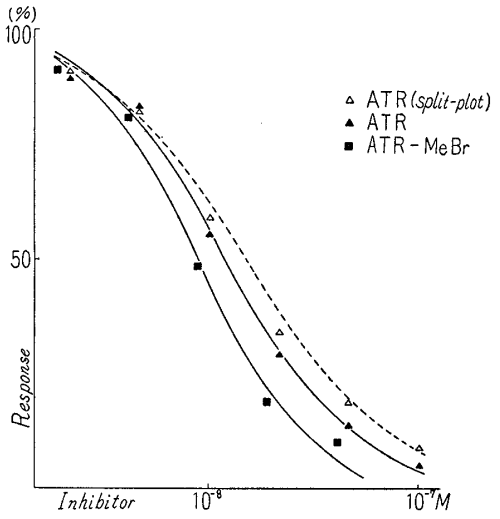

Fig. 3.

Concentration-Inhibition Curves of ACh Contraction (2.2×10^{-6} M) by Atropine and its Methobromide

Discussion

We are now in a position to discuss the various processes concerned in this deter-

mination. On the Atr-dependence of the sensitivity of intestines to ACh, an important factor is the time when Atr is added before administration of ACh. In this experiment, five minutes was adopted as the minimum time to exert Atr action, for shorter exposure to Atr considerably weakens its inhibitory action, though affecting little on the slope of the concentration-action curve of ACh.

At the same time, there are other questions to be considered in this discussion. It follows from the results of Expt. I that longer interval after washing-out of Atr tends to increase the sensitivity of intestines to ACh. Although this reason for the after-effect of Atr is not given satisfactorily, it seems possible that sensitivity of ACh-receptors must be elevated. These results, in the long run, lead us to pay considerable attention in case much time is required in a bioassay.

On the other hand, a general conclusion to be drawn from Table IV is that, as an after-effect, Atr causes little change in the slope of the concentration-action curve of ACh. This apparently indicates that Atr did not exert an essential influence upon ACh-receptor. Even in this antagonistic mechanism, therefore, the law of mass action was supported.

Recently, many studies have been added to the mode of action of Atr to which our considerable attention has been attracted. Although the object of all these studies agrees substantially with ours, there are decided difference of consideration on the antagonistic action. They are concerned with the Gaddum's equation[7] (1), from which we derived equation (2) by an algebraic rearrangment :

$$e^{X_A}=K_A(1+e^{nX_B}/K_B)\frac{y}{y'-y} \qquad (1)$$

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

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

X_A and X_B are log concentration of the active drug A and the antagonist B, y the response by X_A in the presence of X_B, y' the maximum response, and y'' is the response at X_A without the antagonist. Equation (2) is a logistic sigmoid consisting of some concentration levels of Atr against a constant concentration of ACh, from which reaction order of Atr can be estimated in accordance with the method reported previously.[4] Thus, from the facts that the concentration-action curve of ACh shifted parallel to higher doses with the increasing concentration of Atr (cf. Fig. 2) and that the maximum contractions were not lowered in the presence of Atr, it was evident that Atr molecules came into antagonism competitively with ACh, combining with its receptor.

Moreover, it is clear that the slope of concentration-inhibition curve of Atr is regarded as 1.5 in both of two experimental designs (cf. Table X). By the way, the pA method proposed by Schild[8] can be applied only when the antagonism between A and B is competitive and when the reaction orders of two compounds with the receptors are both 1. In the case of ACh-Atr antagonism, it was proved that the reaction order of ACh was 1 and that of Atr was 1.5. If the reaction order of Atr is 1.5, the theoretical value of pA_2-pA_{10} must be $(\log 9)/1.5=0.636$, which agrees fairly well with that obtained experimentally by Timms[9] or by Marshall.[10] In the case of Atr-MeBr, the antagonistic action is thought to be competitive and its reaction order is not significantly different from 1.5.

7) J. H. Gaddum : J. Physiol., **89**, 7(1937).
8) H. O. Schild : Brit. J. Pharmacol., **2**, 189(1947).
9) A. R. Timms : *Ibid.*, **11**, 273(1956).
10) P. B. Marshall : *Ibid.*, **10**, 354(1955).

Recently, Stephenson[11] called our attention to his new receptor theory assuming a factor ε, called efficacy by him, in the drug-receptor combinations. According to his concept, concentration-response relationship of agonists will be determined rather by the contracting systems, situating beyond the receptor-site, than by the interaction between drug and receptor. On the other hand, antagonists was thought by him to exert no influences upon the receptor- and postreceptor-mechanism (i.e. $\varepsilon=0$), so that the deviation of the observed slope (=1.5) in Atr from the theoretical value (=1) can not be elucidated without another assumption.

We prefer to consider that the slope of agonist or of antagonist is determined rather by the lack of parallelism of the concentration between an external and a bio-phase.

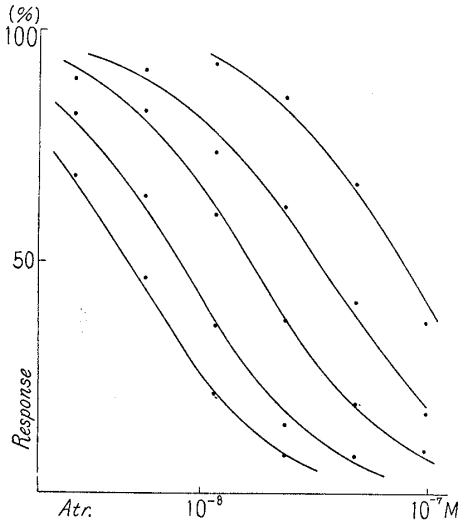

Fig. 4.

Concentration-Inhibition

Curves of Atr

From the equation (2), the position of the inhibition curve of Atr is determined by X_A, that is to say, by the concentration of ACh. Now, by re-plotting curves between responses and Atr doses from Fig. 2, we can see concentration-inhibition curves of Atr to each concentration of ACh in Fig. 4, which brought out the fact that these curves shifted in parallel according to the change of ACh concentration from 6.88×10^{-8} to $7.16\times10^{-5}M$. This point provides a basis for a separatory assay of Atr- and papaverine-like action by ACh in the next report.

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

Summary and Conclusions

(1) The after-effect of Atr tends to increase the contractibility of samll intestines in mice to ACh.

(2) The log concentration-action curves of ACh were obtained under a constant concentration of Atr. There was no depression of the maximum at each Atr. The reaction order n between ACh and its receptor was not changed significantly from 1. These results indicate the competitive nature of antagonism between ACh and Atr.

(3) The estimate of n was not significantly different from 1.5 according to our results on the concentration-inhibition curve of Atr against a constant concentration of ACh.

(4) By means of the log concentration-inhibtion curve of Atr-MeBr, the mode of action was shown to be competitive, the same as Atr, and the estimate of n was not significantly different from 1.5, either. The former has a little more effect than the latter.

(Received June 5, 1957)

11) R. P. Stephenson : *Ibid.*, **11**, 379(1956).