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A QUANTITATIVE METHOD FOR THE ASSAY OF INHIBITORS OF ACID GASTRIC SECRETION IN THE RAT

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Assays of inhibitors of gastric secretion are described by a method of recording continuously acid gastric secretion in the rat. A number of doses of inhibitor comprising several assay blocks can be administered in one preparation and complete assays with known fiducial limits performed with only two rats. Assays were carried out with vasopressin against secretion induced by histamine, and with atropine, methanthelinium and atropine methylnitrate against secretion induced by methacholine.

Methods for the quantitative assay of inhibitors of gastric secretion have been described for the rat and the dog. Visscher, Seay, Tazelaar, Veldkamp, and Vander Brook (1954) and Shea (1956) have described assay methods in the pylorus-ligated rat in which the reduction of spontaneous secretion due to an inhibitory drug is measured. Each rat receives only one dose of inhibitor, but by combining several experiments quantitative results can be obtained. In the pouch dog an essentially similar principle is used (Benjamin, Rosiere, and Grossman, 1950). A submaximal secretion (Code, Hightower, and Hallenbeck, 1951) is induced by an intravenous infusion of histamine, and this is reduced by the inhibitory drug. Although in this assay the same animal can be used repeatedly, only one dose of inhibitor is administered in each test so that a number of experiments must be combined to give a quantitative result.

The present method, which is based on the procedure for continuous recording of gastric secretion described in a previous paper (Ghosh and Schild, 1958), differs from the earlier methods in two main respects. (i) Several doses of inhibitor are administered in succession so that each experiment comprises one or more assay blocks. (ii) The inhibitory drug is given before the stimulant and is thus used to prevent rather than counteract the effect of the latter. In this respect the method resembles the " double histamine " test. The administration of several doses of inhibitor in the same preparation introduces certain complications arising from persistence of the effect of the inhibitor, but it eliminates sources of error due to animal variation and renders the assay considerably more efficient than previous methods in terms of numbers of animals used.

Two inhibitors were studied, vasopressin and atropine. Posterior pituitary hormones are known to inhibit gastric secretion (Dodds, Hills, Noble, and Williams, 1935 ; Gray, Culmer, Wells, and Wieczorowski, 1941 ; Wolf and Wolff, 1947) probably by their vasoconstrictor action (Cutting, Dodds, Noble, and Williams, 1937), and we have found vasopressin to be an effective inhibitor of histamine secretion in rats. The inhibitory effect of vasopressin is readily reversible so that in an assay there is relatively little interference from one dose to the next. The effects of atropine and related compounds, tested against methacholine stimulation, are less readily reversible and tend to be cumulative. This difficulty can be partly overcome by the use of a design in which the dose order is taken into account as recently shown by Rocha e Silva and Rothschild (1956): two atropine-like compounds, methanthelinium (Banthine) and atropine methylnitrate (Eumydrin) were assayed against atropine and it was possible to perform a statistically controlled assay with only two rats for each comparison.

METHODS

The method of Ghosh and Schild (1958) for the continuous recording of acid gastric secretion in the rat was used. Briefly, this method is based on perfusion of the stomach of the rat anaesthetized with

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urethane with a dilute sodium hydroxide solution and graphical registration of the *p*H of the emerging fluid. All drugs were administered intravenously, usually by single injections, in a volume of 0.2 to 0.4 ml. Antagonists were usually injected immediately before the agonists. The following drugs were used: histamine acid phosphate (activity expressed in terms of base), vasopressin (Pitressin), atropine sulphate, atropine methylnitrate (Eumydrin), and methanthelinium (Banthine, Searle).

RESULTS

Effect of Vasopressin on Acid Secretion Induced by Histamine

When an intravenous injection of vasopressin is administered together with or immediately before an intravenous injection of histamine, the normal secretory effect of histamine is inhibited. Fig. 1 shows (on the original circular chart) the

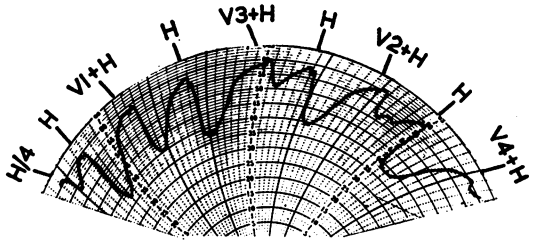

FIG. 1.—Effect of intravenous injections of 200 μg. histamine (H) and 200 μg. histamine with vasopressin (V_1=120 mU., V_2=170 mU., V_3=340 mU., V_4=480 mU.), on *p*H of stomach perfusate. Original circular chart. Distance between horizontal lines=0·5 *p*H units, between solid vertical lines=1 hr. In this and subsequent figures the ordinate represents the *p*H of the stomach perfusate following intravenous injection of drugs in the rat under urethane at 30°.

effect of four doses of vasopressin injected together with histamine, each dose of inhibitor being followed by a dose of histamine alone. The inhibitory effect of vasopressin is graded according to dose. A graded effect could generally be obtained within a four-fold dose range. In Fig. 2*a* the responses from individual experiments are joined, and in Fig. 2*b* linear regression lines are fitted to each set of experimental points; with one exception, the effect increases with dose and the slopes of the regression lines are approximately parallel.

Quantitative Assays with Vasopressin

These assays were planned as 2 + 2 assays with known concentrations of vasopressin. One or more blocks of four doses were administered in the same rat. At the beginning of each experiment, one or two control injections of histamine were given followed by combined injections of a constant dose of histamine with varying doses of vasopressin.

Three experiments with several doses of vasopressin in the same preparation were carried out. In one preparation three randomized blocks of four doses of vasopressin were administered and in the others two blocks of four doses. One of the assays with two blocks is shown in Fig. 3. A dose of 50 μg. histamine was combined with 14, 20, 42, and 60 mU. vasopressin and the experiment was treated as an assay of two solutions of vasopressin. Fig. 4 shows the regression lines for "standard" and "unknown." The activity ratio estimated from these results is 1.32 as against a true ratio of 1.43.

Analysis of variance of this experiment showed that: (i) the *mean square* for *regression* is significant at the 5% level though not at the 1% level; (ii) there is no evidence of deviation from parallelism; (iii) there is no significant difference between the two assay blocks. In the other two experiments similar results were obtained. The *mean*

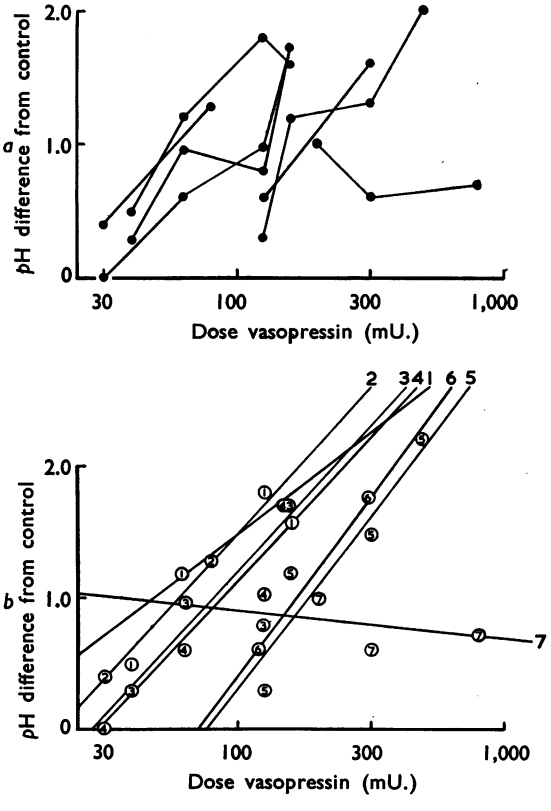

FIG. 2.—Dose/effect curves for vasopressin. The ordinate represents reduction of secretory effect by histamine+vasopressin as compared with histamine alone. In the upper panel responses from individual experiments are joined. In the lower panel calculated regression lines were fitted. Numerals in circles correspond to regression lines.

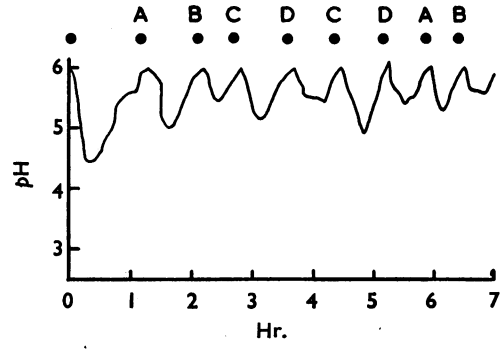

FIG. 3.—Two blocks of 4 doses of vasopressin injected together with a constant dose of histamine (50 μg.). A=20 mU.; B=60 mU.; C=14 mU.; D=42 mU. vasopressin.

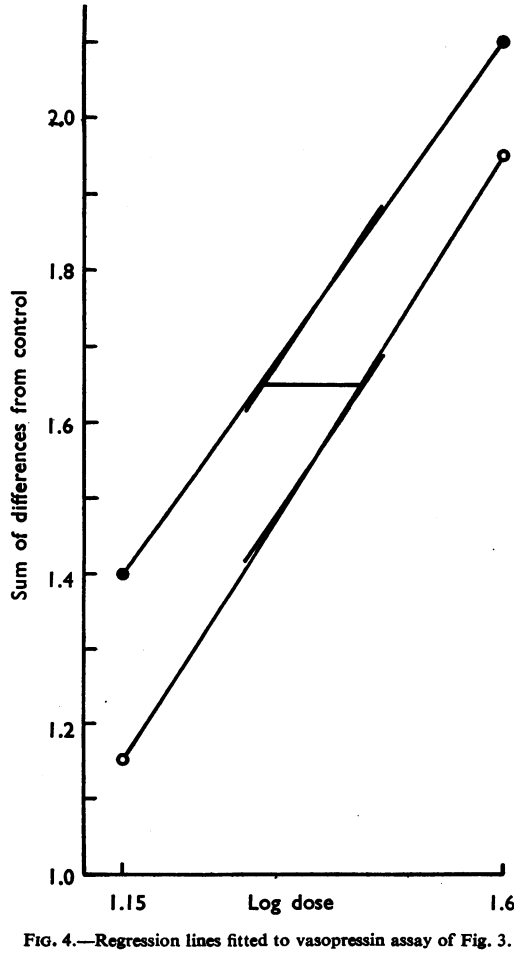

FIG. 4.—Regression lines fitted to vasopressin assay of Fig. 3.

square for *regression* was significant at $P<0.05$ in one assay and at $P<0.01$ in the other. There was no significant deviation from parallelism and no significant difference between successive assay blocks. In another series a dose of histamine was interpolated between doses of vasopressin to allow for more complete recovery from inhibition. This series involved three rats, each of which received four doses of vasopressin, the combined results being treated as a single assay. In the analysis of variance regression was highly significant; there was no evidence of deviation from parallelism. Differences between assay blocks were near-significant. Since each set of four doses was administered to a different animal, differences between assay blocks were to be expected. In conclusion, then, all the assays gave a satisfactory regression and no deviation from parallelism. The true and estimated activity ratios and the values for λ in the four assays are shown in Table I. The λ value expresses the

TABLE I

TRUE AND ESTIMATED ACTIVITIES AND λ VALUES OF FOUR ASSAYS WITH HISTAMINE AND VASOPRESSIN

Expt. No.	% Activities		λ
	True	Estimated	
194 (without recovery dose) ..	125	137	0·26
195 ,, ,, ,, ..	143	132	0·16
197 ,, ,, ,, ..	130	121	0·16
201, 202, 203 (with recovery dose) ..	140	224	0·11

intrinsic precision of the experiment. All assays gave reasonable λ values: the lowest value (highest precision) was obtained in the preparations which were allowed to recover between doses of inhibitor.

Effect of Atropine-like Substances on Methacholine-stimulated Secretion

Three experimental designs were employed to assay atropine-like substances against methacholine: 1. Continuous infusion of methacholine and inhibition by intermittent doses of atropine. When methacholine was administered by slow constant infusion in doses of 10 to 40 μg./hr., it produced a continuous stimulation of acid secretion which could be counteracted by injections of atropine. Fig. 5 shows the effects of intravenous injections of doses of atropine ranging from 0.03 to 0.3 μg. in a preparation stimulated by constant infusion of methacholine. Although graded effects could be obtained in this way with atropine the responses were irregular, and after large doses

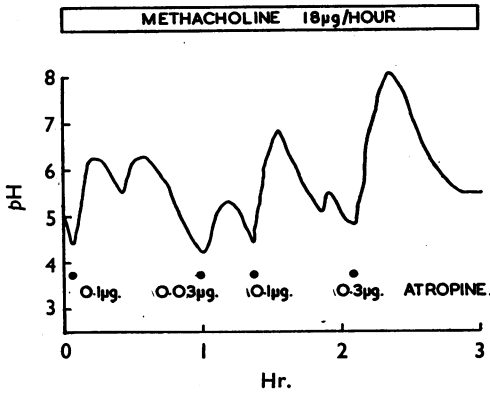

FIG. 5.—Inhibition of acid secretion by single intravenous doses of atropine superimposed on a slow intravenous infusion of methacholine.

the curve failed to return to the base line. Furthermore, continuous intravenous injection of methacholine produced considerable bronchial secretion, dyspnoea, and other toxic effects. 2. Intermittent injections of methacholine and atropine; dose-ratio assay. Single intravenous injections of methacholine produce a transient secretory response graded according to dose (Ghosh and Schild, 1958). Atropine reduces this response and the antagonism can be measured in terms of the dose of antagonist required to reduce the effect of a double dose of methacholine to that of a single dose. (This corresponds to a pA_2 determination, except that the antagonist is measured as a dose instead of as a concentration.) An experiment of this kind is shown in Fig. 6. Methacholine (2.5 μg.) produced a deflexion of about 1 *p*H unit, whereas 5 μg. produced a larger deflexion. Atropine (0.2 μg.) caused a slight reduction of the effect of 5 μg. methacholine and 0.4 μg. a larger reduction. By graphical extrapolation, the dose of atropine which reduced the effect of methacholine to that of half the dose was estimated as 0.47 μg. Table II shows other estimates of this quantity with methacholine and carbachol used as a stimulant. The activity of homatropine tested in this way was about 20 times less than that of atropine. 3. Intermittent injections of methacholine and two atropine-like substances. In these experiments constant doses of methacholine were combined with varying doses of atropine-like drugs. The experiments were carried out as 2 + 2 assays in a Latin square design. In one assay, consisting of a comparison of methanthelinium and atropine by a 4 × 4 Latin square design, only two rats were used, each receiving two blocks of four doses. Within each block consecutive doses of antagonist were administered, but recovery doses of methacholine were interpolated between the blocks. The results of this assay are shown in Table III and one of the two experiments is illustrated in Fig. 7. The ratio between large and small doses of inhibitor was three, and each time the larger dose produced a greater inhibition than the smaller dose. The analysis of variance of this assay (Table IV) gave highly significant F values for regression and for difference between rats. The F value for *dose order* does not quite reach the 5% level, but it is sufficiently large to suggest that position in the block is important. Fig. 8 shows that the regression lines for the two antagonists are parallel. The activity ratio for methanthelinium and atropine was 10.7 with 5% fiducial limits of 8.2 and 14.1.

TABLE II
DOSE OF ANTAGONIST REQUIRED TO REDUCE EFFECT OF AGONIST TO THAT OF HALF THE DOSE

Stimulant	Antagonist	Antagonist Dose (μg.)
Methacholine	Atropine	0·76
,,	,,	0·47
Carbachol	,,	0·72
Methacholine	Homatropine	12·5

TABLE III
RESULTS OF THE 2+2 ASSAY OF METHANTHELINIUM AND ATROPINE

Drug	Dose (μg.)	Maximum Deflexion of *p*H in Different Groups				Total
Atropine ..	1·0	1·4	0·9	1·9	1·9	6·1
	3·0	0·3	0·25	1·1	0·45	2·1
Methanthelinium	0·1	0·9	0·8	2·35	1·8	5·85
	0·3	0·3	0·2	0·55	0·7	1·75
Sum of groups ..		2·9	2·15	5·9	4·85	15·8

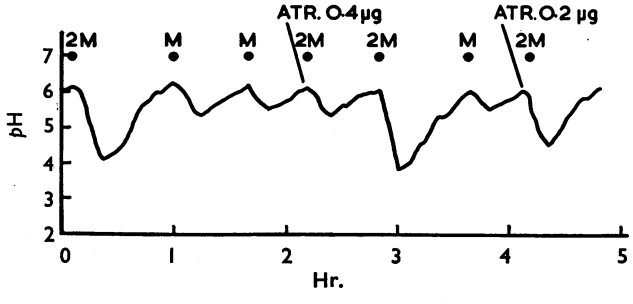

FIG. 6.—Inhibition of methacholine-induced secretion by simultaneous injection of graded doses of atropine.

This result is almost the opposite of that reported by Benjamin *et al.* (1950), who estimated the activity ratio of methanthelinium and atropine against histamine secretion in the dog (Table VII). In view of this discrepancy the comparison was repeated, this time with one rat for each block and with doses of methacholine after each dose of inhibitor. This assay gave an activity ratio of 10.7 (fiducial limits 4.4 to 29.1 at 5% level), thus confirming the previous results. The λ values and fiducial limits in this and the previous assays are shown in Table V. The λ value of the second methanthelinium assay was considerably larger than that of the first, suggesting that the more time-consuming method involving recovery doses had no special advantages in this instance. In another assay a comparison of atropine methylnitrate and atropine was carried out. This assay involved two rats, one of which

TABLE IV

ANALYSIS OF VARIANCE OF THE RESULTS OF THE ASSAY OF METHANTHELINIUM AND ATROPINE

Source of Variation	Sum of Squares	d.f.	Mean Square	F	
Between rats	2	1	2	50	$P<0.01$
Between groups	0·25	2	0·12	3	$P>0.05$
Dose order	0·5	3	0·17	4·25	$P>0.05$
Between "standard" and "unknown"	0·02	1	0·02		
Regression	4·1	1	4·1	100·25	$P<0.01$
Deviation from parallelism	0·001	1	0·001		
Error	0·23	6	0·04		
Total ..	7·101	15			

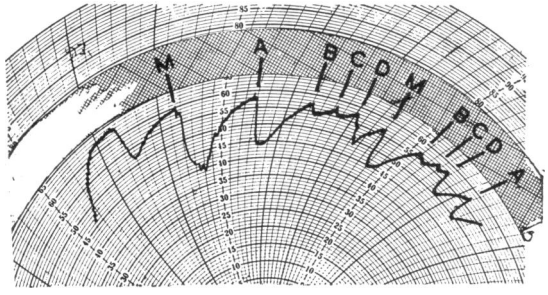

FIG. 7.—Part of Latin square assay of methanthelinium and atropine. The antagonists were injected together with a constant dose of methacholine (5 μg.). M=methacholine 5 μg.; A=atropine 1 μg.+M; B=atropine 3 μg.+M; C=methanthelinium 0·3 μg.+M; D=methanthelinium 0·1 μg.+M. Each large horizontal division corresponds to 0·5 *p*H units.

TABLE V

ESTIMATED ACTIVITY, FIDUCIAL LIMITS AND λ VALUE OF THREE ATROPINE-LIKE DRUGS

Assay	Activity Ratio	Fiducial Limits (P=0·95)	λ
Methanthelinium/atropine (without recovery dose). 2 rats	10·7	8·2–14·1	0·09
Methanthelinium/atropine (with recovery dose). 4 rats	10·7	4·4–29·1	0·25
Atropine methylnitrate/atropine (without recovery dose). 2 rats	3·1	2·0–4·8	0·14

TABLE VI

ANALYSIS OF VARIANCE OF THE RESULTS OF THE ASSAY OF ATROPINE METHYLNITRATE AND ATROPINE

Source of Variation	Sum of Squares	d.f.	Mean Square	F	
Between groups	1·18	3	0·39	11·2	$P<0.01$
Dose order	0·28	3	0·09	2·7	$P>0.05$
Between "standard" and "unknown"	0·002	1	0·002		
Regression	1·69	1	1·69	48·2	$P<0.01$
Deviation from parallelism	0·09	1	0·09	2·6	$P>0.05$
Error	0·21	6	0·035		
Total ..	3·452	15			

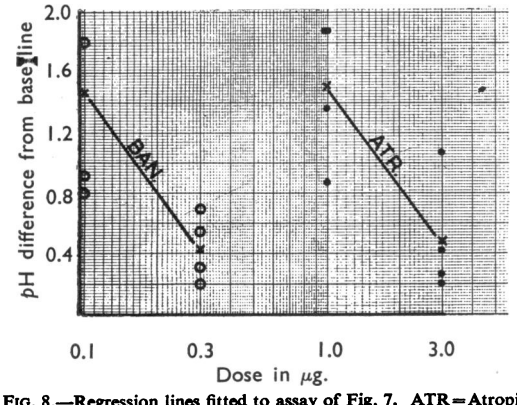

FIG. 8.—Regression lines fitted to assay of Fig. 7. ATR=Atropine; BAN=Methanthelinium.

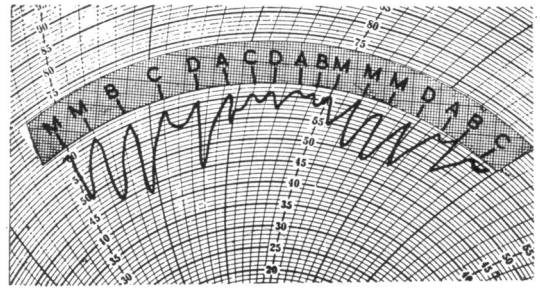

FIG. 9.—Part of assay of atropine and atropine methylnitrate. Three blocks of 4 doses injected in the same rat. M=methacholine 10μg.; A=atropine methylnitrate 0·3 μg.+M; B=atropine 0·3 μg.+M; C=atropine 0·9 μg.+M; D=atropine methylnitrate 0·1 μg.+M.

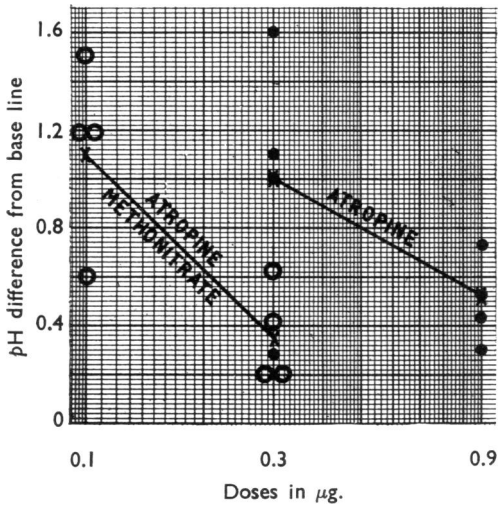

FIG. 10.—Regression lines fitted to assay of Fig. 9.

received one block and the other three blocks of four doses. The latter part of the assay is illustrated in Fig. 9. It shows a cumulative effect of successive doses of inhibitor which at first sight would seem to invalidate the result. Analysis of variance, however, shows that the result is nevertheless valid, since the F value for regression is highly significant and there is no significant deviation from parallelism (Fig. 10) (Table VI). The activity ratio of atropine methylnitrate and atropine was 3.1 with fiducial limits 2.0 and 4.8 at the 5% level.

DISCUSSION

The object of this work has been to devise statistically valid assays for inhibitors of gastric secretion making use of the continuous perfusion method described in an earlier paper (Ghosh and Schild, 1958). In this paper only inhibitors with a transient action are discussed. Assays of long-lasting inhibitors of the urogastrone type will be discussed elsewhere (Ghosh, Gregory, and Schild, unpublished observations).

Vasopressin is an example of a short-lasting inhibitor whose effect is probably due to vasoconstriction. A series of doses of vasopressin constituting one or more assay blocks can be given in the same preparation with relatively little interference from one dose to the next, although the more consistent results (lower λ values) obtained by interpolating doses of histamine suggest that interference between successive doses of inhibitor is not completely eliminated. But lower λ values have to be balanced against loss of time, and, in this sense, the method with the interpolated doses of histamine is less efficient.

Atropine-like drugs produce more persistent effects than vasopressin, but it is nevertheless possible to administer repeated doses in one preparation and perform a satisfactory assay. Estimates of relative activity and fiducial limits could be obtained by the use of only two rats for a complete assay. In spite of interaction between successive doses the dose/response relation of the inhibitor is not obliterated; in each experiment the larger dose produced a significantly greater effect than the smaller. A Latin square design was adopted in order to equalize dose order and thus correct to some extent for persistence of effects. This design seemed justified, since in the analysis of variance the mean square for dose order considerably exceeds error; although the F ratio does not reach the 5% level of significance.

In terms of number of animals required to achieve a given degree of accuracy the present assay method is much more efficient than the assay method in the pylorus-ligated rat. The efficiency of an assay can be expressed by n/λ^2 where n is the number of doses administered to one animal. In the present assays average values for n and λ are 8 and 0.16. No λ values have been published for the pylorus-ligation method, but a rough estimate on the basis of the published results of Visscher *et al.* (1954) gives a value of $\lambda = 0.85$ with $n = 1$. The ratio of efficiencies of the two methods thus works out at 190.

TABLE VII
ACTIVITY RATIOS FOR SECRETORY INHIBITORS

Inhibitor	Administration	Stimulant	Preparation	Activity Ratio	Reference
Methanthelinium/atropine ..	Subcutaneous	Histamine	Gastric secretion (dog)	1/20	Benjamin *et al.* (1950)
	Intravenous	Spontaneous secretion	Gastric secretion (pylorus-ligated rat)	1/10	Visscher *et al.* (1954)
	,,	Methacholine	Gastric secretion (rat stomach)	10	Present work
	,,	Carbachol	Salivary secretion (cat)	2·2	Johnson and Wood (1954)
Atropine methyl iodide/atropine	Intramuscular	Spontaneous secretion	Gastric secretion (pylorus-ligated rat)	10	Shea (1956)
Atropine methylnitrate/atropine	Intravenous	Methacholine	Gastric secretion (rat)	3·1	Present work

Such calculations have of course only limited validity, since different assay methods are not interchangeable and may give widely different results, especially when different stimulants are used. This is shown in Table VII, which gives the activity ratios for methanthelinium-atropine and methylatropine-atropine as secretory inhibitors when tested by different quantitative methods.

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