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THE AFFINITY AND EFFICACY OF ONIUM SALTS ON THE FROG RECTUS ABDOMINIS

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It has long been known that, whereas tetramethylammonium ions (Me_4N^+) stimulate nicotine-sensitive acetylcholine receptors, tetraethylammonium ions (Et_4N^+) block them. The early work is reviewed by Raventós (1937), who himself made a systematic survey of the effects of the step-wise replacement of methyl groups in Me_4N^+ by ethyl groups. On the frog rectus he found that activity declined eight-fold when one methyl group was replaced by ethyl, and that further replacement gave compounds which showed neither agonist nor antagonist activity in the highest concentrations tested. Marshall (1916), on the other hand, had found that the compounds dimethyldiethylammonium ($Me_2N^+Et_2$), methyltriethylammonium (MeN^+Et_3) and Et_4N^+ antagonized the contracture produced by tetramethylammonium on the frog sartorius muscle. This would seem to indicate that the compounds still combine with the receptors, so the loss in activity must be due to a loss of efficacy. None of the results, however, give any quantitative indication of the effect of the replacement of methyl by ethyl on the affinities of these compounds for the receptors and we have, therefore, re-examined them together with their pyrrolidine, piperidine, quinuclidine and pyridine analogues. The blocking actions of ions as similar as these are likely to be competitive, and we have measured the affinity constants of the antagonists and partial agonists for the nicotine-sensitive acetylcholine receptors in the frog rectus muscle, as well as the relative activities of those of the compounds which are agonists.

METHODS

The frog rectus preparation

The rectus abdominis muscle from *Rana pipiens* was used in all experiments. It was mounted in frog-Ringer solution, through which air was blown, and kept at room temperature (around 15° C). Contractions were recorded with an isotonic gimbal-mounted lever, writing on a smoked drum. The load was between 0.5 and 1.0 g. The volume of the bath was approximately 5 ml., but this is not important because all drugs were made up to the desired concentration in frog-Ringer. This solution flowed from a reservoir on to the tissue at the appropriate time, determined by opening and closing a magnetic relay, operated by a time-clock and uniselector. In all the experiments the agonist was in contact with the tissue for 4½ min. It was then washed out and the preparation gently stretched (by increasing the load on the lever by about 5 g) for 15 min, during which period it was again washed twice. It was then left with the extra load removed and the next application of

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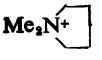
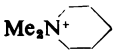
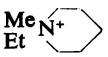
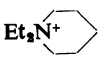
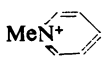
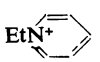
agonist was made exactly 30 min after the previous one. This long time-cycle was necessary because the recovery of the tissue after drugs such as tetramethylammonium is very much slower than after acetylcholine. Regular responses were obtained over periods of 24 to 48 hr, however, when the rectus was treated in this way.

Compounds

All the compounds were prepared by standard methods and the analytical results are shown in Table 1. Methylquinuclidinium iodide and ethylquinuclidinium iodide were made by Dr. Jamieson Walker.

TABLE 1

ANALYTICAL RESULTS

Compound	m.p.	% Iodide Found	% Iodide Theory
Me_4N^+	>350°	63·2	63·2
Me_3N^+Et	dec. 310°	59·1	59·0
$Me_2N^+Et_2$	dec. 304°	55·4	55·4
MeN^+Et_3	307–8°	52·2	52·3
Et_4N^+	dec. 300°	49·4	49·4
	350°	55·8	55·9
	314–5°	52·7	52·6
	300–1° dec.	49·9	49·7
	350°	52·6	52·6
	302–3° dec.	50·0	49·8
	281–2° dec.	47·2	47·2
	117–8°	57·7	57·5
	84–5°	54·2	54·0

All the compounds are iodides and the analyses are for ionized iodine, determined gravimetrically. Melting-points were determined on a Kofler hot stage and are uncorrected. The compounds were recrystallized from combinations of methanol, ethanol, and methylethyl ketone.

The relative activity of agonists

All the compounds which stimulated the preparation were compared with Me_4N^+ in 2+2 dose assays with four repetitions of each dose. If the slopes of the log. dose-response lines were the same, the result of the comparison was expressed as an equipotent molar ratio—that is, as the number of molecules of the compound producing the same effect as one molecule of Me_4N^+. As in the bioassay of two solutions of the same drug, this ratio was calculated from the average slope of the log. dose-response lines and the average difference in the size of the responses. Each drug was tested on a number of preparations and the values of the logarithm of the equipotent molar ratio were expressed as a mean and standard deviation.

The log. dose-response lines of some of the compounds were clearly flatter than those of Me_4N^+; these substances were found to be partial agonists and their affinity constants were measured by the methods described below.

The affinity constants of antagonists

These were measured exactly as described by Barlow, Scott & Stephenson (1963) for antagonists of acetylcholine at the muscarine-sensitive acetylcholine receptors of the guinea-pig ileum. Tetramethylammonium was used as the agonist. On each preparation the dose-ratios were measured for two (or more) concentrations of antagonist and from these, two (or more) values of the affinity constant were obtained. The mean of these was taken as the value for the preparation. Each compound was tested on a number of preparations and the result was expressed as the mean value of the logarithm of the affinity constant and the standard deviation.

Affinity constants of partial agonists

The affinity constants of most of the partial agonists were measured by two methods. Method I is that described by Stephenson (1956). Method II is described below.

Responses were obtained with a number of concentrations of the partial agonist and of a potent agonist (Me_4N^+ was used in all these experiments). The concentrations producing the same response were then compared. If the same response is produced by a concentration a of the potent agonist, which has an affinity constant K_A and an efficacy e_A, and by a concentration p of the partial agonist, which has an affinity constant K_P and an efficacy e_P, the biological stimulus, S, should be the same in both circumstances—that is,

$$\frac{e_A a K_A}{1+aK_A} = \frac{e_P p K_P}{1+pK_P}$$

If the agonist has a high efficacy and the proportion of receptors occupied, y, is small.

$$aK_A = \frac{y}{1-y} \rightarrow y$$

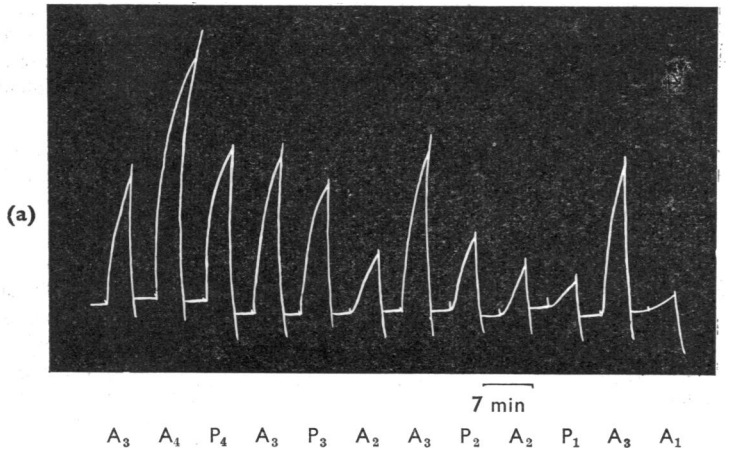
$$\text{so } S = e_A a K_A = \frac{e_P p K_P}{1+pK_P}$$

$$\text{and } \frac{1}{a} = \frac{e_A K_A}{e_P K_P}\frac{(1)}{p} + \frac{e_A K_A}{e_P}$$

and the graph of $1/a$ against $1/p$ should be a straight line with an intercept, when $1/a=0$, or $-K_P$.

In all experiments, responses were obtained with three concentrations of Me_4N^+ and with three, sometimes four, concentrations of the partial agonist, which produced responses intermediate between those obtained with Me_4N^+ (Fig. 1a). By a procedure similar to that used in a three-point assay with two concentrations of standard and one of unknown, the concentration of Me_4N^+ was calculated which should produce the same response as a particular concentration of partial agonist. The reciprocal of the concentration of partial agonist was then plotted against the reciprocal of the concentration of Me_4N^+ which had been calculated to produce the same response. The best line

which fitted the points, judged by eye, was extrapolated to obtain K_P (Fig. 1b). Each partial agonist was tested on a number of preparations and the results were expressed as the mean value of the logarithm of the affinity constant and the standard deviation.



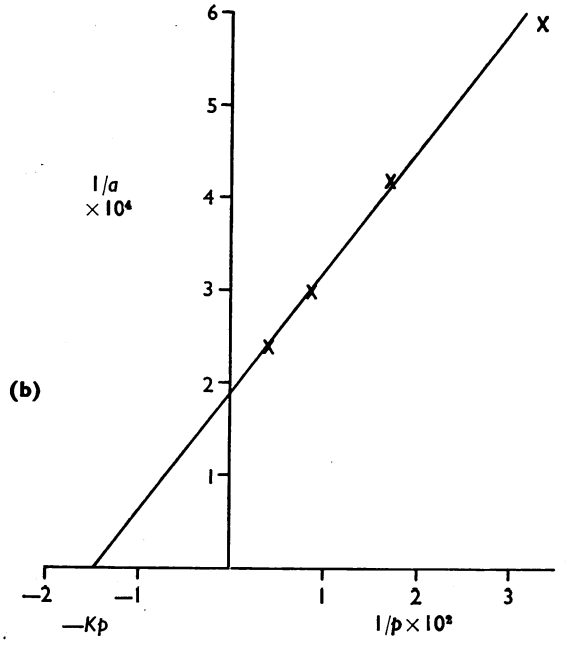


Fig. 1. Measurement of the affinity constant of a partial agonist. a. Responses of the frog rectus to concentrations of tetramethylammonium, 1, 2, 4, and 8×10^{-5}M (A_1, A_2, A_3, and A_4 respectively), and to methylpyridinium, 3, 6, 12, and 24×10^{-5}M (P_1, P_2, P_3, and P_4 respectively). The compounds were left in contact with the tissue for 4½ min. The kymograph was stopped for 23 min during the washing and recovery period, so the interval between doses was 30 min. b. Graph of $1/p$ against $1/a$ calculated from the results shown in Fig. 1*a*; $K_P=1.5\times10^3$.

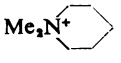
RESULTS

Table 2 shows the logarithms of the equipotent molar ratios of the agonists relative to Me_4N^+ ; the mean value is shown, together with the standard deviation and the number of tissues on which the assay was made. The Table also shows the logarithms of the affinity constants of the partial agonists and antagonists, again as the mean and standard deviation.

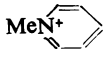
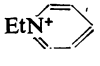
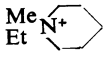
TABLE 2

EFFECTS ON THE FROG RECTUS

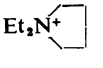
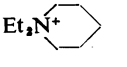
A. Ability to cause contracture: log. equipotent molar ratio relative to Me_4N^+:

	0·320±0·032 (10)
Me_3N^+Et	0·437±0·026 (10)
Methylquinuclidinium	0·738±0·022 (5)
	0·826±0·054 (7)
$Me_2N^+Et_2$	1·018±0·019 (5)
	1·049±0·078 (9)

B. Partial agonists: log. affinity constant:

	Method I	Method II	Pooled
MeN^+Et_3	2·39±0·28 (6)	2·56±0·09 (3)	2·45± 0·24 (9)
	2·64±0·29 (5)	2·49±0·23 (9)	2·54± 0·25 (14)
	2·63±0·16 (4)	2·36±0·09 (4)	2·49± 0·19 (8)
	2·87±0·19 (5)		
Ethylquinuclidinium	3·02±0·42 (3)	3·03±0·23 (6)	3·02± 0·28 (9)

C. Antagonists: log. affinity constant:

Et_4N^+	2·87±0·15 (4)
	3·24±0·11 (3)
	3·49±0·15 (5)

Method I is that described by Stephenson (1956), Method II is that described in this paper. Values are the mean of the number of estimates shown in parentheses ± the standard deviation.

DISCUSSION

It is quite striking that the variance of the estimates of the log. affinity constant in Table 2 is far greater than the variance of the estimates of the log. equipotent molar ratio. Each estimate of the latter, however, is based on a number of comparisons in a single experiment, whereas each experiment for measuring affinity constants usually gives only one comparison in a single experiment, because the log. dose-reponse line in the absence of the antagonist is only obtained once, at the beginning of the experiment. There do not appear to be previous reports of the variance of estimates of the log. affinity constant of antagonists for acetylcholine receptors in the frog rectus, but the variance of our estimates appears to be slightly higher than that obtained with antagonists on the acetylcholine receptors of the guinea-pig ileum by Schild (1947) and much greater than that obtained by Barlow *et al.* (1963).

The variance of the estimates of the log. affinity constant of the partial agonists is greater than for the pure antagonists. The results do not suggest any reason for preferring one or other of the two methods used. The first involves the calculation of the proportion of receptors, x, occupied by a partial agonist when present in a concentration, p, and the affinity constant,

$$K_P = \frac{x}{1-x}\left(\frac{1}{p}\right),$$

consequently any error in the measurement of x affects both the numerator and denominator in opposite directions and will lead to larger differences in the estimate of K_P. One source of variation with the second method is the need for extrapolation in order to obtain K_P. The mean values of the estimates obtained by the two methods are not significantly different except with ethylpyridinium. The values of the log. affinity constant of this compound are significantly different at a 5% level of probability, but not at a 1% level. The variance of these estimates, however, is appreciably lower than that of the other compounds and the difference would not be significant at a 5% level if the variance were the same as for methylpyridinium, MeN^+Et_3, or ethylquinuclinium. It seems likely that the difference arises from a sampling error and does not indicate any real difference between the values obtained by the two methods.

The results confirm and extend the findings of Raventós (1937) and Marshall (1916) that replacement of methyl groups in Me_4N^+ by other groups leads to a decline in activity and the appearance of antagonism. Figures 2a and 2b indicate two general tendencies; with increased ionic weight there is a decrease in potency, and an increase in affinity. If one looks at all the compounds the effects are not very great, but within groups, where the increase in ionic weight is due to replacement of a methyl group by an ethyl group, the effects are quite clear. In all three instances in which a methyl group is replaced by ethyl in the agonists, and in two instances out of three in the antagonists, the activity is altered by a factor of about 3. Where the increase in ionic weight is brought about by inserting a methylene group to expand a ring from pyrrolidinium to piperidinium, the effects are again in the same direction, but the fall in potency from dimethylpyrrolidinium to dimethylpiperidinium is greater than the rise in affinity from diethylpyrrolidinium to diethylpiperidinium.

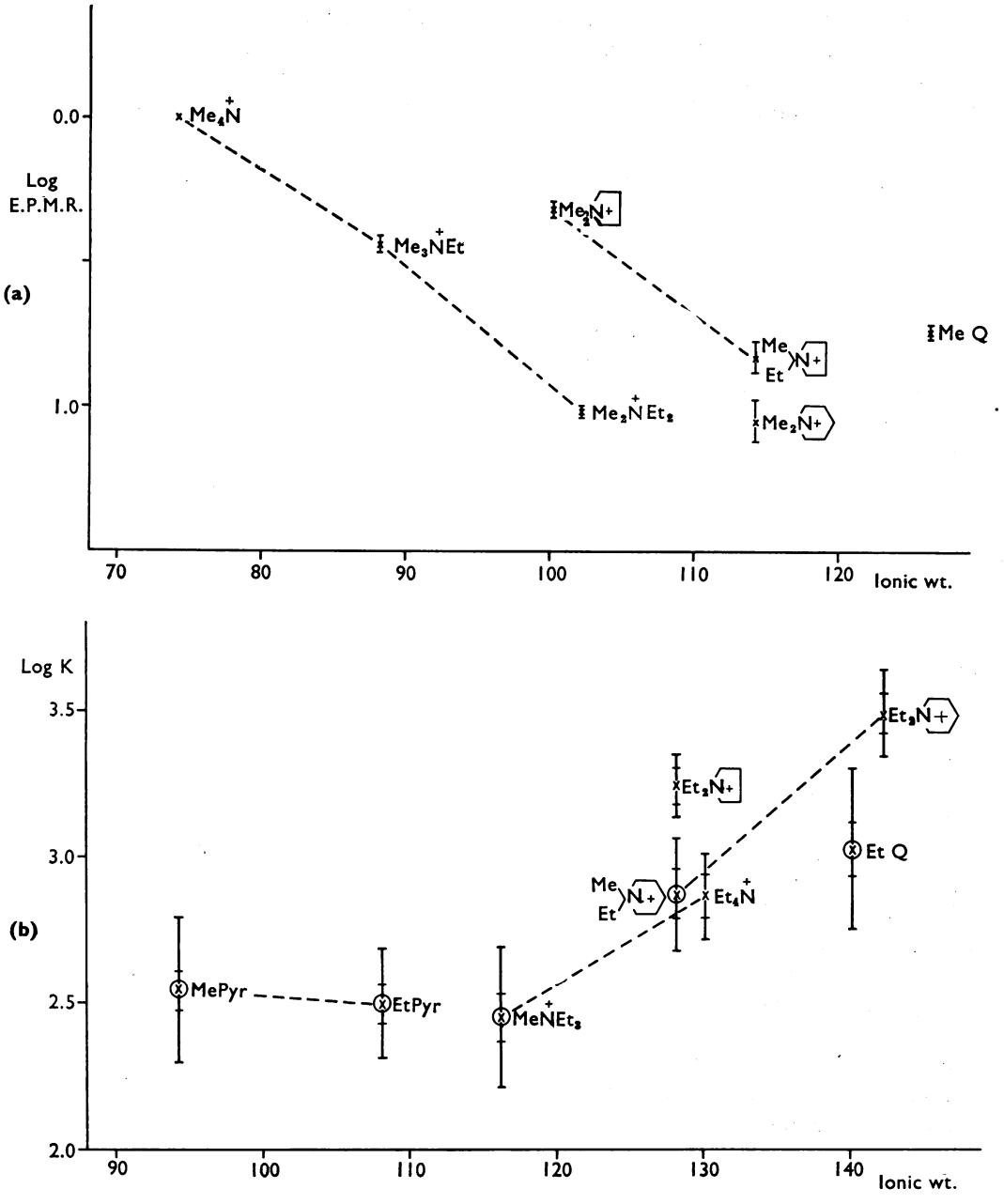


Fig. 2. Graphs of ionic weight against: (a) a the log. equipotent molar ratio of the agonists relative to tetramethylammonium. Note that the scale has been inverted so that the most active compounds appears at the top of the picture. The vertical bars indicate the standard deviation. b. the log. affinity constant of the partial agonists (⊗) and antagonists (×). The vertical bars indicate the standard deviation and the inner marks the standard error. The lines join compounds in the same series. MeQ=methylquinuclidinium ; EtQ=ethylquinuclidinium ; MePyr=methylpyridinium ; EtPyr=ethylpyridinium.

The two trends shown in Figs. 2a and 2b make it clear that an increase in ionic weight leads to a reduction in efficacy. This is particularly clear when the reduction is such that the compounds become partial agonists or antagonists and no longer appear on the same graph. For example, $Me_2N^+Et_2$ appears in Fig. 2a whereas MeN^+Et_3, being a partial agonist, appears in Fig. 2b, as does Et_4N^+, which is a pure antagonist.

If it is possible to extrapolate the observed change in affinity per methylene group from the antagonists and partial agonists in a series to the agonists (for which it cannot be measured directly), it follows that the general decrease in activity by a factor of 3 per methylene group (Fig. 2a), and increase in affinity by a factor of about 3 (Fig. 2b), indicate a decrease in efficacy by a factor of about 9 per methylene group.

In general the change from agonist to partial agonist with these simple compounds takes place in the range of ionic weights from 110 to 120, and from partial agonist to antagonist in the range 120 to 130. Methylquinuclidinium, however, with an ionic weight of 126, is still a true agonist and ethylquinuclidinium is still a partial agonist, although it has an ionic weight of 140. Methylpyridinium, on the other hand, with an ionic weight of only 94, has such a low efficacy that it is only a partial agonist. Clearly ionic weight is not the sole influence. Quinuclidinium compounds appear to have lower affinity and more potency and efficacy than would be expected, whereas methylpyridinium has unexpectedly high affinity and low efficacy. The quinuclidine ring system, however, is very compact and it is likely that the size of the ion is more important than the ionic weight. The shape of the ion, too, is likely to affect its properties, and it is notable that the ions with flat rings, methylpyridinium and the pyrrolidinium compounds, appear to have higher affinities than would be expected. In contrast ethylquinuclidinium, which has a lower affinity than might be expected, cannot assume a flat conformation.

Stephenson (1956) suggested that, in general, high affinity for acetylcholine receptors in the guinea-pig ileum was not compatible with high efficacy, and the present results indicate that the same may be true for the acetylcholine receptors of the frog rectus. The pyrrolidinum compounds, however, appear to possess relatively high affinity and have also high activity, so have retained a high efficacy. An incompatibility between high affinity and high efficacy is readily explained by "rate theory" (Paton, 1961; Paton & Rang, 1966), and the effect of an extra methylene group, discussed above, would thus be to reduce the rate of combination of drug with receptor, k_1, by a factor of 3 and to reduce the rate of dissociation, k_2, by a factor of 9. This would account for the increase in affinity, K $(=k_1/k_2)$, by a factor of 3 and the decrease in efficacy by a factor of 9. But there is no direct evidence for this and the complete absence of "fade" with the rectus, the responses of which are still increasing at the end of $4\frac{1}{2}$ min, makes it unlikely that the details of Paton's theory can apply in this situation.

SUMMARY

1. Series of compounds obtained by replacing methyl groups by ethyl in tetramethylammonium (Me_4N^+), dimethylpyrrolidinium, dimethylpiperidinium, methylpyridinium and methylquinuclidinium have been tested on the frog rectus abdominis preparation.

2. Tetramethylammonium, ethyltrimethylammonium (Me_3N^+Et), dimethyldiethylammonium ($Me_2N^+Et_2$), dimethylpyrrolidinium, methylethylpyrrolidinium, dimethyl-

piperidinium and methylquinuclidinium ions are all agonists and cause contracture. Tetraethylammonium (Et_4N^+), diethylpyrrolidinium, and diethylpiperidinium are antagonists. Methyltriethylammonium (MeN^+Et_3), methylethylpiperidinium, methylpyridinium, ethylpyridinium and ethylquinuclidinium are partial agonists. The activities of the agonists relative to tetramethylammonium have been estimated and the affinity constants of the antagonists and partial agonists have been measured.

3. Except with the pyridinium compounds, the progressive replacement of methyl groups by ethyl leads to a rise in affinity in a series and a loss in agonist activity, presumably due to loss in efficacy. To some extent the changes can be related to the increase in ionic weight, but the size and shape of the ion is also important. The pyrrolidinium and pyridinium compounds, with flat rings, have a higher affinity than might be expected from their ionic weight alone, whereas ethylquinuclidinium has a lower affinity. Methylpyridinium has a low efficacy and is a partial agonist, whereas pyrrolidinium and quinuclidinium compounds have a relatively high efficacy.

4. The relationship between efficacy and affinity is discussed.

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