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THE KINETICS OF COMPETITIVE ANTAGONISTS ON GUINEA-PIG ILEUM

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1 The kinetics of action of some competitive muscarinic and histamine antagonists were examined on guinea-pig isolated ileum and their behaviour compared with the predictions of the interaction-limited model described by Paton (1961).

2 The kinetics of antagonism were not consistent with the predictions of this model: (1) The apparent dissociation rate constant calculated from the decrease in occupancy on washout was not independent of the concentration of antagonist. (2) The dissociation rate constant of a 'slow' antagonist calculated from the change in occupancy when a 'fast' antagonist was superimposed varied with the concentration of fast antagonist. (3) If the concentration of slow antagonist was increased when the fast antagonist was superimposed so that the equilibrium occupancy of the 'slow' was the same as before, a transitional phase was observed.

3 The kinetics of antagonism were observed in longitudinal muscle strips and intact pieces of ileum, bathed in Tyrode or Krebs solution, and with isometric and isotonic recording. No evidence was found that the discrepancies between the interaction-limited model and the observed kinetics could be accounted for by the experimental method used.

4 It is therefore concluded that either access is rate-limiting in these circumstances or, if interaction is rate-limiting, some alternative interaction-limited model is required to describe the kinetics of antagonism. In either case it would seem unwise at this time to calculate antagonist-receptor rate constants from the observed kinetics of antagonism.

Introduction

If the mathematical model derived by Paton (1961) based on a simple mass action relationship describes how a competitive antagonist combines with its receptors, the affinity constant, K, of the antagonist will be the ratio of two constants, k_1 and k_{-1}, the rate constants describing the rate of association and dissociation from the receptors. Furthermore, if the kinetics of antagonism on an isolated tissue is limited by the rate at which the antagonist interacts with the receptors, values of k_{-1}, (and hence k_1 as $K = k_1/k_{-1}$), could be calculated from the observed kinetics of antagonism and differences in affinity between antagonists could be related to differences in k_1 and k_{-1} separately (Paton, 1961; Paton & Rang, 1965).

Despite apparent agreement with this model in certain circumstances (Paton, 1961; Paton & Rang, 1965; Rang, 1966; Ginsborg & Stephenson, 1974) certain serious discrepancies have led to the suggestion that it is the rate of access to the receptors which governs the kinetic behaviour of antagonists (Thron & Waud, 1968). The following study was therefore undertaken to provide further evidence as to whether access to the receptor or interaction with the receptor normally limits the kinetics of competitive antagonists on guinea-pig ileum and hence whether it is reasonable to calculate rate constants from the observed kinetics. Part of this work has been communicated to the Pharmacological Society (Roberts & Stephenson, 1974).

Methods

Intact pieces of ileum or longitudinal muscle strips were prepared from 150 to 400 g guinea-pigs as described by Edinburgh Staff (1968) and Paton & Rang (1965). The preparations were suspended in an organ bath containing a bathing solution at 36°C. Usually a Tyrode solution bubbled with air and of the following composition was used: (mM) Na^+ 149.2, K^+ 2.7, Mg^{2+} 1.1, Ca^{2+} 1.8, Cl^- 143.2, HCO_3^- 11.9, $H_2PO_4^{2-}$ 0.4, SO_4^{2-} 1.1, glucose 5.6 and 27.6 hexa-

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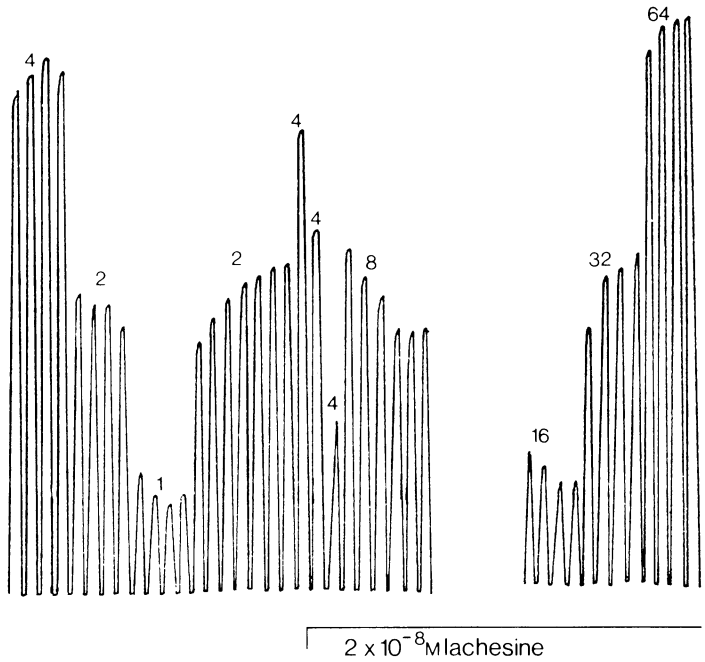

Figure 1 The onset of action of lachesine illustrating the method used to follow the kinetics of antagonism. There is a gap in the trace of 28.5 minutes. The paper drive to the potentiometric recorder was on from just before the agonist was added to just after washout. The concentrations of agonist used are indicated above the responses — M x 10^7, (i.e. $4 = 4 \times 10^{-7}$ M).

methonium bromide. Alternatively a Krebs solution bubbled with 95% O_2 and 5% CO_2 was used: (mM) Na^+ 155.8, K^+ 5.6, Mg^{2+} 1.1, Ca^{2+} 2.2, Cl^- 162.1, HCO_3^- 1.79, glucose and 10^{-7} M atropine.

Events in the organ bath were controlled by automatic apparatus and the responses were recorded in an analogue and digital form (Stephenson, 1968; Abramson, Barlow, Mustafa & Stephenson, 1969). The fluid in the bath was changed at the appropriate time by upward displacement and overflow with the bathing solution, either alone or containing drug(s) at predetermined concentrations. The agonist was in contact with the tissue for 17 s before being washed out twice, with a 30 s interval between the washings. The agonist (either carbachol or histamine as appropriate) was applied every 90 seconds.

In most experiments the responses produced by the agonist were recorded with an isotonic lever with a differential transformer as a transducer. A weight of between 0.5 and 0.8 g was used to load the lever when intact pieces of ileum were used, and between 0.2 and 0.5 g when muscle strips were used. Alternatively, where specified, an isometric transducer was used (Devices Physiological Transducer 2.S.T.O.2).

The organ bath was connected via a two-way stopcock to either of two sets of 5 reservoirs, 4 containing agonist and one without. Turning the stopcock enabled a quick change to be made from a set of solutions not containing antagonist to a set all containing antagonist or *vice versa*. Similarly a change could be made from a set with one antagonist to a set with two.

Initially three concentrations of agonist were used as shown in Figure 1, the responses being within the 'linear' region of the log dose-response curve. When the tissue had settled down, usually in about 2–3 h, one concentration of agonist was repeated a number of times. The stopcock was then turned, usually during a contraction so that the peak of the following contraction occurred 90 s after the change. The establishment of the new equilibrium was followed by changing the concentrations of agonist applied so that the response size remained within a narrow range.

To follow the kinetics of onset and offset of antagonism the tissue was set up in the absence of antagonist and a log dose-response curve plotted from the last sequence of responses before the antagonist was added. A certain concentration of antagonist was then introduced and the dose-ratio, DR_t, corresponding to each response during onset determined from the curve and the corresponding antagonist occupancy p_t calculated from the formula

$P_t = (DR_t - 1)/DR_t$. When equilibrium was established the antagonist was removed and offset followed in a similar way.

Occupancy changes during each experiment were plotted using the convention of Paton & Rang (1965). Values of $(p_\infty - p_t)$ or p_t during onset or offset respectively were plotted on a log scale against time. If the relationship between log occupancy and time appeared to be reasonably linear time constants for the development and decline in occupancy were determined from the slopes of straight lines drawn through the points by eye. In those experiments where there did not appear to be a reasonably linear relationship, time constants corresponding to the initial rate were calculated from the first three responses (for lachesine or antazoline) or from the first response (for pentyl triethyl ammonium iodide (pentyl TEA)).

When studying the offset of benziloyltropine methyliodide (BTrMe) on superimposition of pentyl TEA, the tissue was initially equilibrated with a certain concentration of BTrMe. The fast antagonist was then superimposed and the change in dose-ratio followed. The offset was not followed to equilibrium in all experiments.

A log dose-response curve was plotted from the last sequence of responses before the changeover and dose-ratios corresponding to each subsequent response determined relative to this curve. BTrMe's occupancy corresponding to the response at time t after the changeover, $p_{S,t}$, was calculated from this dose-ratio in the following way: If A_S and $A_{S+F,t}$ are the concentrations of agonist required to produce the same response in the presence of the slow antagonist alone and in the presence of slow and fast at time t after the changeover, the observed dose-ratio DR_t will be equal to $A_{S+F,t}/A_S$. If a is the concentration of agonist which would have been required to produce the same response in the absence of antagonists and $A_{S+F,t}/a$ is called $DR_{F+S,t}$ and A_S/a is called DR_S: $DR_t = A_{S+F,t}/A_{SL} = DR_{F+S,t}/DR_S$. $DR_{F+S,t}$ is equal to the reciprocal of the proportion of free receptors, i.e. $DR_{F+S,t} = 1/(1 - p_{S,t} - p_F)$ and similarly $DR_{SL} = 1/(1 - p_S) = (1 + c_S K_S)$. Therefore the observed dose-ratio

$$DR_t = 1/[(1 - p_{S,t} - p_F)(1 + c_S K_S)].$$

If it is further assumed that the fast antagonist is at all times in equilibrium with the receptors not occupied by the slow, $p_F = c_F K_F (1 - p_{S,t})/(1 + c_F K_F)$. Therefore knowing c_F, K_F, K_S and the observed dose-ratio DR_t, BTrMe's occupancy at time t, $p_{S,t}$ can be calculated.

The value of K_F used was 3.6×10^4 M^{-1}. This corresponds to the mean of 24 estimations using concentrations of pentyl TEA between 0.3×10^{-4} M and 8×10^{-4} M (mean ± s.e. mean, $3.69 \pm 0.15 \times 10^4$ M^{-1}). This value agrees with that obtained by Abramson *et al.* (1968), (Log $K = 4.588$; $K = 3.87 \times 10^4$ M^{-1}). However, when higher concentrations of pentyl TEA were examined the mean dose-ratio was lower than expected. For instance the mean dose-ratio produced by 30×10^{-4} M was 53.7 ($n = 6$) and not around 110 as expected. The reason for this change in apparent affinity is not clear as it did not appear to be associated with a change in slope of the log dose-response curve and was not more marked if agonists such as pentyl or hexyl trimethylammonium (both lower efficacy than carbachol) were used instead of carbachol. Pentyl TEA was originally selected as the fast antagonist in our experiments because it was the weakest readily available and so potentially the fastest; potent antagonists such as atropine with affinities around 10^9 M^{-1} and upwards are very slow whereas faster antagonists have lower affinities than this. However, the kinetics of some antagonists, such as desoxylachesine, with affinities in the 10^7–10^8 M^{-1} range, were subsequently found to act just as fast as the less potent pentyl TEA. It would therefore have been better to use a more potent antagonist than pentyl TEA, not only to avoid the anomalous dose-ratios produced by high concentrations but also to avoid the very large amounts of pentyl TEA used.

The value of K_S used was 1.5×10^{10} M^{-1}. This corresponds to the mean of 27 estimations using concentrations of BTrMe between 10×10^{-10} and 200×10^{-10} M (mean ± s.e. mean = $1.55 \pm 0.17 \times 10^{10}$ M^{-1}). This is somewhat lower than the value of 2.36×10^{10}M^{-1} obtained by Barlow & Mustafa (1968), but with these values of K_S and K_F the antagonists produced the expected combined dose-ratios even when concentrations of pentyl TEA greater than 8×10^{-4} M were used.

For each experiment values of $(p_{S,t} - p_{S,\infty})$ were plotted on a log scale against time and as there was a reasonably linear relationship, time constants were determined from the slopes of the straight lines drawn through the points by eye.

As lachesine equilibrates so much more rapidly than BTrMe it was possible to follow first the onset of action of lachesine and then its offset on superimposition of pentyl TEA. Values of $p_{S,t}$ were calculated as above but using the equilibrium dose-ratios produced in that experiment by lachesine alone and in combination with pentyl TEA to estimate $(1 + c_S K_S)$ and $(1 + c_F K_F)$. The value of $(1 + c_F K_F)$ was consistent with the equilibrium dose-ratios produced by pentyl TEA alone even when high concentrations of pentyl TEA were used. This is therefore different, for some unknown reason, from the combination of pentyl TEA with BTrMe. Values of $(p_{S,t} - p_\infty)$ were plotted as before but τ_{off} was calculated from the first three responses after the changeover since the relationship between log occupancy and time was not reasonably linear. The offset of lachesine on superimposition of desoxylachesine and octyl TMA was followed in a similar way.

The following drugs were kindly provided by Dr R.B. Barlow: benziloyltropine methyliodide (BTrMe); diphenylhydroxyacetoxyethyl dimethylethylammonium bromide (lachesine); diphenylacetoxyethyl dimethylethylammonium iodide (desoxylachesine); *n*-octyl trimethylammonium iodide (octyl TMA); *n*-pentyl triethyl ammonium iodide (pentyl TEA). Other drugs used were: antazoline hydrochloride (Ciba), atropine sulphate (BDH), carbaminoylcholine chloride (carbachol, BDH), hexamethonium bromide (Koch-Light), mepyramine maleate (May and Baker).

The following notation is used:

c, c_S, c_F the concentration of a drug, of a slow antagonist and of a fast antagonist (M).

p_o, p_t, p_∞ the proportion of receptors occupied by a drug initially, after a time t and at equilibrium.

p_S, p_F the proportion of receptors occupied by a slow antagonist and a fast antagonist.

DR_t, DR_∞ the dose-ratio produced by an antagonist at time t, and at equilibrium.

DR_{F+S}, DR_F, DR_S the combined dose-ratio produced by a fast and a slow antagonist acting together, by a fast alone, and a slow alone, all at equilibrium.

K, K_S, K_F the affinity constant of an antagonist, of a slow antagonist and of a fast antagonist (M^{-1}).

k_1, k_{-1} the antagonist receptor association and dissociation rate constants ($s^{-1}M^{-1}$, s^{-1}).

τ_{on}, τ_{off} the time constant describing an increase or decrease in occupancy (s or min).

I The kinetics of onset and offset of antagonism

Paton (1961) observed that, contrary to the predictions of the interaction-limited model, the rates of offset from high concentrations of atropine and mepyramine were not independent of the antagonist concentration and he suggested that this was due to appreciable intracellular uptake. These antagonists have therefore been re-examined together with quaternary compounds with which it was hoped to minimize this complication.

Theory

If the rate of association and dissociation from the receptors determines the rate of increase or decrease of an antagonist's occupancy when it is added or removed from the bathing solution surrounding an isolated tissue then, (Paton, 1961), during onset:

$$p_t = p_\infty(1 - \exp[-(k_1 c + k_{-1})t])$$

and during offset

$$p_t = p_o \exp[-k_{-1} t]$$

Therefore

1. The antagonist's occupancy should change exponentially during both the onset and offset of antagonism.
2. As the time constant for offset τ_{off} is equal to $1/k_{-1}$ it should be independent of concentration.
3. The ratio τ_{off}/τ_{on} should be equal to the equilibrium dose-ratio.

If access were rate-limiting the predicted behaviour would depend on the particular access model being considered.

Results (I)

As shown in Figure 2 and Table 1 the kinetics of antagonism do not appear to be consistent with the interaction model. In particular, following the onset from higher concentrations of BTrMe the rate of offset became faster and following higher concentrations of atropine, antazoline, lachesine, mepyramine and pentyl TEA the rate of offset became slower.

The increasing rate of recovery following exposure to higher concentrations of BTrMe is surprising as, at any time after about 10 min from the beginning of the washout period, the tissue is actually more sensitive to carbachol after having been treated with a high than a low concentration of BTrMe. As this was not observed when longitudinal muscle strips were used instead of intact pieces of ileum (Section IV, Table 5), the kinetics of BTrMe on intact pieces of ileum was reinvestigated subsequently and again the same observation was made.

These differences cannot be attributed to a change in affinity as significant differences in apparent affinity were not observed except with pentyl TEA (see Methods section). With pentyl TEA the rates of offset became appreciably slower at concentrations well below that at which this change in affinity was observed and so cannot be totally attributed to it.

In addition these discrepancies cannot be explained by intracellular uptake as they are not restricted to the non-quaternary compounds, as the change in rate of offset of BTrMe was not associated with a decrease in the time to which the tissue was exposed to the antagonist (about 3 h) and also increasing the time to which the tissue was exposed to 1×10^{-4} M pentyl TEA from 9 to 30 min did not appear to influence the subsequent rate of offset.

Again, contrary to the predictions of the interaction limited model, some of the plots in Figure 2 do not appear to be linear but in view of the unavoidable changes in the sensitivity of the tissue with time, non-linearity of semilogarithmic plots for antagonists as slow as BTrMe is difficult to interpret. However, pentyl TEA and antazoline are so fast that the curvature of such offset plots can hardly be attributed to a change in sensitivity. The non-linearity of the onset plots with BTrMe and lachesine may also be an artifact as the time taken for equilibrium to be established was considerably less than that predicted from the

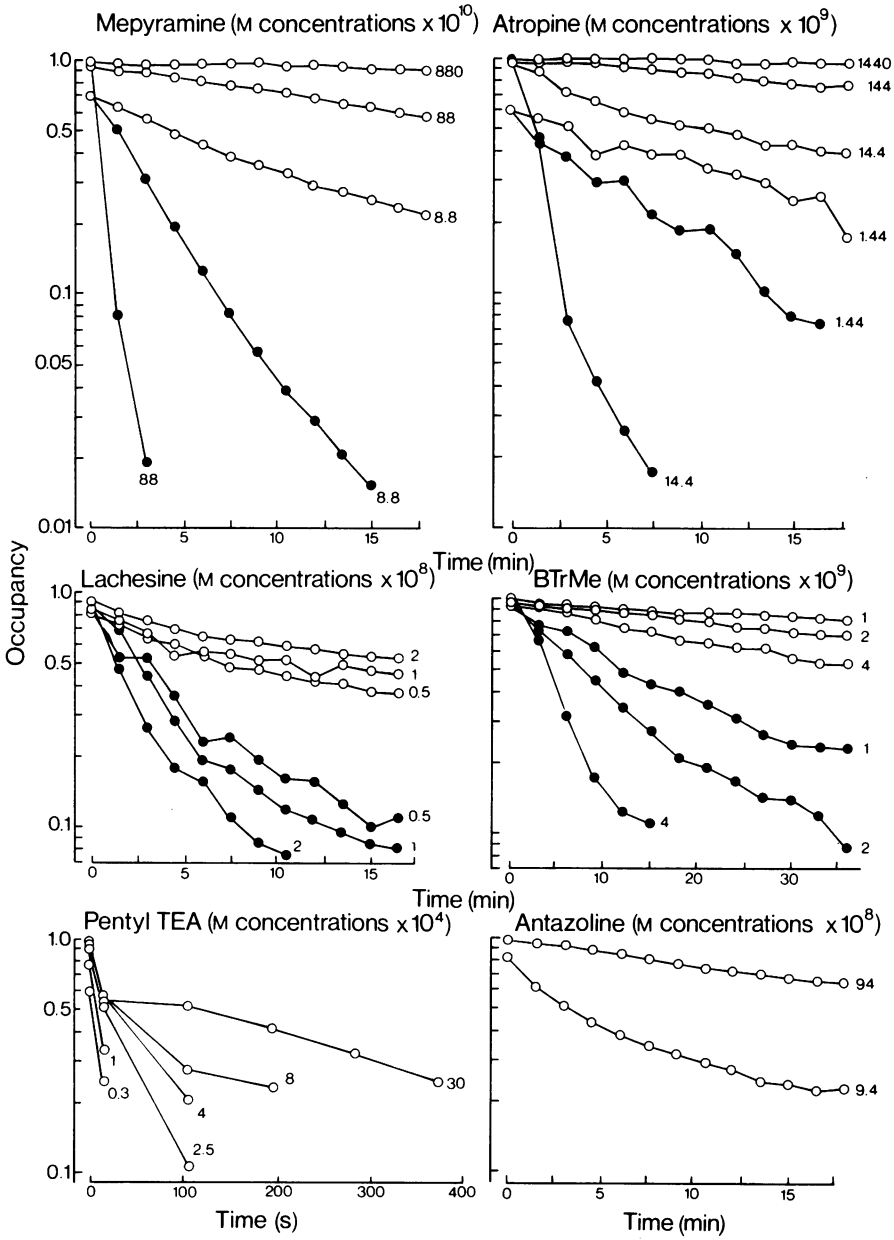

Figure 2 The kinetics of onset and offset of six antagonists illustrating the relationship found between occupancy plotted on a log scale and time. The mean occupancy at the time corresponding to each response was calculated from the results of the individual experiments in each group (Table 2). These values are plotted using the convention of Paton & Rang (1965): for onset (closed circles) values of $(p_\infty - p_t)$, and for offset (open circles) values of p_t were plotted on a log scale against time. If interaction were rate-limiting there would be a linear relationship between occupancy plotted on a log scale and time. Also τ_{off} would be constant for each antagonist. BTrMe = benziloyltropine methyliodide; Pentyl TEA = *n*-pentyl triethyl ammonium iodide.

occupancy plots. This may be due to the way in which the equilibrium occupancy was obtained. In these experiments, after an initial period during the onset using one concentration of agonist, three were then used until equilibrium appeared to have been established. This could be held to have produced, by desensitization or some such effect, a larger apparent equilibrium dose-ratio than would otherwise have been obtained and this would have induced curvature into the onset plots.

II The 'displacement' of a slow antagonist on superimposition of a fast

Thron & Waud (1968) suggested that if access rather than interaction is rate-limiting the large concentration gradient resulting when a potent slow antagonist is 'displaced' from the receptors on superimposition of a fast antagonist might accelerate the removal of the slow antagonist from the tissue as compared to simply washing out the slow antagonist. This prediction has been investigated.

Theory

If interaction were rate-limiting and a fast antagonist superimposed on a tissue previously equilibrated with a very much slower antagonist, the fast antagonist can be considered to equilibrate instantaneously with the receptors not occupied by the slow. This would cause the 'displacement' of the slow antagonist according to the following equation (Rang, 1966):

$$p_{S,t} = p_{S,o} - (p_{S,\infty} - p_{S,o})$$
$$(\exp[-k_{-1}t(1 + c_S K_S + c_F K_F)/(1 + c_F K_F)]$$

and the fast antagonist would be at equilibrium at all time with the receptors not occupied by the slow.

If access were rate-limiting, although the predicted behaviour would depend on the particular model, the values of k_{-1} obtained in this way might be expected to be larger than those obtained in I.

Table 1 The kinetics of onset and offset of six antagonists. (Values are mean ± s.e. mean).

n	*Concentration of antagonist (M)*	*Time constant for onset (τ_{on}) (min)*	*Time constant for offset (τ_{off}) (min) *(s)*	τ_{off}/τ_{on}	*Equilibrium dose-ratio (DR_∞)*
Atropine					
1	1.44×10^{-9}	7.5	17	2.3	2.6
1	14.4×10^{-9}	1	20	20	21.4
3	144×10^{-9}		45 ± 2		355 ± 3.2
3	1440×10^{-9}		437 ± 93		3044 ± 62
BTrMe					
6	1×10^{-9}	27 ± 5	398 ± 82	15 ± 2	16 ± 1
5	2×10^{-9}	12 ± 2	120 ± 7	13 ± 2	36 ± 4
3	4×10^{-9}	6 ± 1	70 ± 5	12 ± 2	60 ± 2
Lachesine					
5	0.5×10^{-8}	6.7 ± 0.9	25.5 ± 2.7	4.0 ± 0.7	4.8 ± 0.4
7	1×10^{-8}	5.7 ± 0.4	22.3 ± 4.4	4.0 ± 0.7	7.4 ± 0.8
16	2×10^{-8}	3.7 ± 0.3	25.6 ± 3.0	7.6 ± 1.0	14.9 ± 1.0
Pentyl TEA					
4	0.3×10^{-4}		*17 ± 4		2.8 ± 0.5
3	1×10^{-4}		*18 ± 2		4.4 ± 0.2
4	2.5×10^{-4}		*33 ± 9		11.1 ± 0.6
4	4×10^{-4}		*44 ± 12		14.2 ± 0.4
5	8×10^{-4}		*36 ± 8		27.9 ± 2.8
4	30×10^{-4}		*59 ± 17		49.4 ± 5.2
Antazoline					
6	9.4×10^{-8}		6.6 ± 0.4		5.8 ± 0.4
6	94×10^{-8}		42.5 ± 2.7		40.7 ± 1.6
Mepyramine					
6	8.8×10^{-10}	3.5 ± 0.1	13.4 ± 0.8	4.0 ± 0.3	3.5 ± 0.1
10	88×10^{-10}	0.6 ± 0.01	35.9 ± 3.3	59.2 ± 6.4	26.5 ± 1.6
4	880×10^{-10}		207 ± 28		215 ± 19

BTrMe = benziloyltropine methyliodide; pentyl TEA = *n*-pentyl triethyl ammonium iodide.

* If interaction were rate limiting, τ_{off} would be equal to $1/k_{-1}$ and so would be constant. Also τ_{off}/τ_{on} would be equal to DR_∞.

Results (II)

Although the offset of BTrMe's occupancy on superimposition of pentyl TEA was reasonably exponential (Figure 3) the rate of offset was accelerated as compared with simply washing out BTrMe (Table 2). The degree of acceleration increased with the concentration of pentyl TEA, approaching a limiting value of about 20 min (Figure 4).

The offset of lachesine's occupancy on superimposition of pentyl TEA was not considered to be sufficiently exponential for time constants to be calculated from the mean slope of the semilogarithmic plots (Figure 5). Time constants corresponding to the initial rate were therefore calculated from the first three responses following the superimposition of pentyl TEA. These correspond to rates faster than would be predicted from the rate of recovery on removal of lachesine from the bathing solution (Figure 6 and Table 1). The limiting value of k_{-1} for lachesine shown in Figure 6 is remarkably similar to that calculated by Paton & Rang (1965) from the rate of offset on removal of lachesine from the bathing solution. It is thus possible that some condition in Paton & Rang's experiments which we were unable to

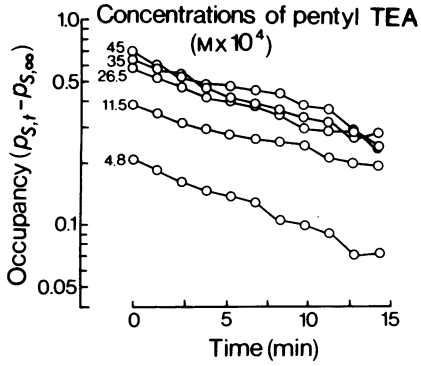

Figure 3 The kinetics of offset of benziloyltropine methyliodide (BTrMe, 4×10^{-9} M) when various concentrations of *n*-pentyl triethyl ammonium iodide (pentyl TEA) were superimposed. The mean occupancy of BTrMe at the times corresponding to each response ($p_{S,t}$) were calculated from the individual experiments in each group (Table 2). The values of $(p_{S,t} - p_{\infty})$ are plotted on a log scale against time. If BTrMe's interaction with the receptors were rate-limiting there would be a linear relationship between occupancy plotted on a log scale against time.

Table 2 The kinetics of offset of benziloyltropine methyliodide (BTrMe) on superimposition of *n*-pentyl triethyl ammonium iodide (pentyl TEA). (Values are mean ± s.e. mean).

n	*Concentration of BTrMe (c_S)* (M)	*Concentration of pentyl TEA (c_F)* (M)	$\tau_{off}\left[\frac{1 + c_S K_S + c_F K_F}{1 + c_F K_F}\right]$ (min)
5	0.6×10^{-9}	2.2×10^{-4}	136.4 ± 23.4
4		2.5×10^{-4}	100.0 ± 26.7
4		5.4×10^{-4}	95.5 ± 17.2
4		10.3×10^{-4}	63.0 ± 8.4
5		25.0×10^{-4}	33.2 ± 6.7
4	1×10^{-9}	1.3×10^{-4}	148.5 ± 22.0
4		2.5×10^{-4}	124.5 ± 21.3
5		3.3×10^{-4}	132.4 ± 18.8
4		6.0×10^{-4}	119.3 ± 16.3
4		7.8×10^{-4}	85.3 ± 8.4
6		14.0×10^{-4}	48.7 ± 5.8
4		25.0×10^{-4}	30.5 ± 7.8
11	2×10^{-9}	2.5×10^{-4}	84.2 ± 8.4
9		6.0×10^{-4}	113.7 ± 16.7
6		14.0×10^{-4}	48.0 ± 5.5
8		25.0×10^{-4}	43.3 ± 7.2
5	4×10^{-9}	4.8×10^{-4}	78.4 ± 19.7
5		11.5×10^{-4}	53.0 ± 8.1
5		26.5×10^{-4}	29.8 ± 4.5
4		35.0×10^{-4}	23.8 ± 3.8
3		45.0×10^{-4}	21.3 ± 0.9

If interaction were rate-limiting, $\tau_{off}(1 + c_S K_S + c_F K_F)/(1 + c_F K_F)$ would be equal to $1/k_{-1}$ and so would be constant.

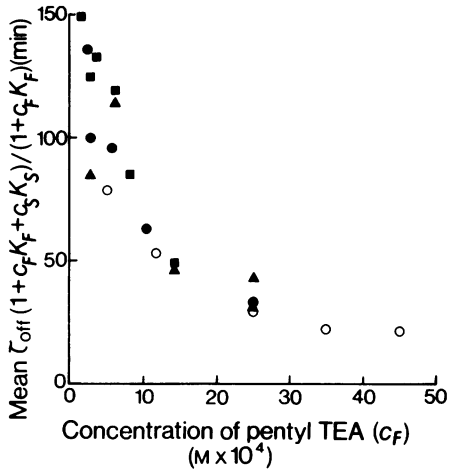

Figure 4 The relationship between the rate of offset of benziloyltropine methyliodide (BTrMe) on superimposition of *n*-pentyl triethyl ammonium iodide (pentyl TEA) and the concentration of pentyl TEA. Mean values of $\tau_{off}(1 + c_SK_S + c_FK_F)/(1 + c_FK_F)$ (Table 2) are plotted against c_F. The initial concentrations of BTrMe used, c_S are indicated as follows: (●) 6×10^{-10} M; (■) 10×10^{-10} M; (▲) 20×10^{-10} M; (○) 40×10^{-10} M. If BTrMe's interaction with the receptors were rate-limiting $\tau_{off}(1 + c_FK_F + c_SK_S)/(1 + c_FK_F)$ would be equal to $1/k_{-1}$ and so would be constant and independent of c_F.

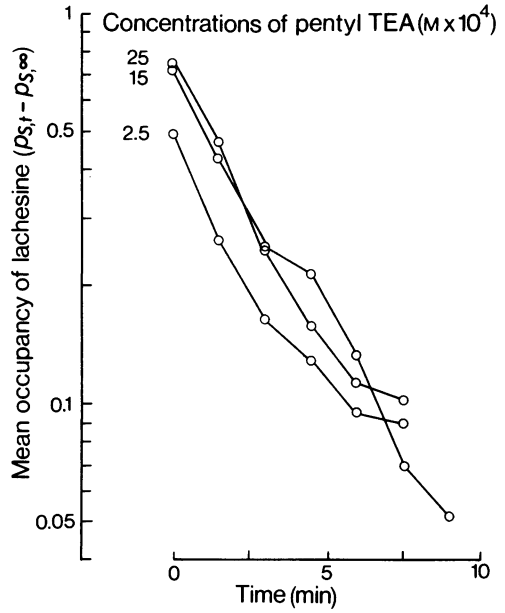

Figure 5 The kinetics of offset of lachesine (1×10^{-8} M) when various concentrations of *n*-pentyl triethyl ammonium iodide (pentyl TEA) were superimposed. The mean occupancy of lachesine at the time corresponding to each response was calculated from the results of the individual experiments in each group (see Figure 6). Mean values of $(p_{S,t} - p_{S,\infty})$ are plotted on a log scale against time. If lachesine's interaction with the receptors were rate-limiting there would be a linear relationship between occupancy plotted on a log scale against time.

reproduce had the effect of greatly reducing the delay in drug access.

In view of the anomalous dose-ratios produced by high concentrations of pentyl TEA, the rates of offset of lachesine on superimposition of the different fast antagonists octyl TMA or desoxylachesine, were observed. These appeared to combine with lachesine as expected for two competitive antagonists:

$$DR_{F+S} = DR_F + DR_S - 1 = 1 + c_SK_S + c_FK_F.$$

The rates observed (Table 3) are consistent with those obtained with pentyl TEA and so there is no reason to believe that the acceleration observed can be attributed to anomalous behaviour of pentyl TEA.

Table 3 The rate of offset of lachesine (2×10^{-8} M) on superimposition of *n*-octyl trimethylammonium iodide (octyl TMA) or desoxylachesine

n	*Fast antagonist*	*Concentration of fast antagonist* (c_F) *(M)*	$\tau_{off}\left[\frac{1 + c_SK_S + c_FK_F}{1 + c_FK_F}\right]$ *(min)*	*Observed dose-ratio*	*Calculated dose-ratio* $\left[\frac{1 + c_SK_S + c_FK_F}{1 + c_SK_S}\right]$
4	Octyl TMA	8.9×10^{-4}	8.1 ± 2.7	3.5 ± 0.6	4.9
4	Octyl TMA	15×10^{-4}	4.3 ± 1.5	7.8 ± 1.0	7.6
4	Desoxylachesine	$.4 \times 10^{-4}$	2.6 ± 0.5	10.1 ± 0.9	11.9

It was assumed that for lachesine $K_S = 6.6 \times 10^8$ M^{-1}, (the mean value of $K \pm$ s.e. mean of 26 estimations was $6.6 \pm 0.4 \times 10^8$ M^{-1}), for octyl TMA $K_F = 6.3 \times 10^4$ M^{-1} (Stephenson, 1956), and for desoxylachesine $K_F = 4 \times 10^7$ M^{-1}, (the mean value of $K \pm$ s.e. mean of 16 estimations was $4.0 \pm 0.3 \times 10^7$ M^{-1}).

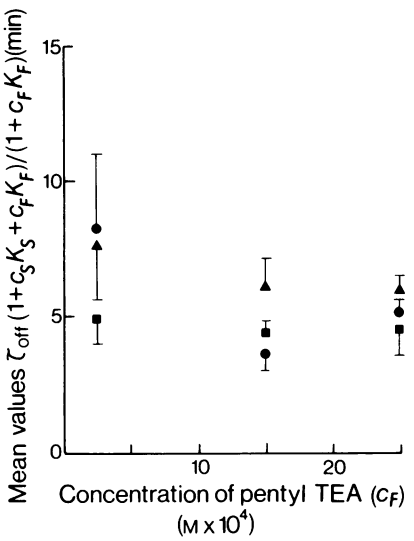

Figure 6 The relationship between the rate of offset of lachesine on superimposition of various concentrations of *n*-pentyl triethyl ammonium iodide (pentyl TEA), and the concentration of pentyl TEA. Mean values are shown ($n=3$). Vertical lines show s.e. mean. The initial concentrations of lachesine, c_S are indicated as follows: (●) 0.5×10^{-8} M; (■) 1×10^{-8} M; (▲) 2×10^{-8} M. If interaction were rate-limiting $\tau_{off}(1 + c_S K_S + c_F K_F)/(1 + c_F K_F)$ would be equal to $1/k_{-1}$ and so would be independent of c_F.

The calculations assume that the fast antagonist is so much faster than the slow that it can be considered to be in equilibrium at all times with the receptors not occupied by the slow. As the onset of all the concentrations of pentyl TEA examined were too fast to be measured this assumption is considered reasonable.

III The interaction between BTrMe and pentyl TEA

The interaction between BTrMe and pentyl TEA was also followed with a slightly different procedure from that in II. As before the tissue was initially equilibrated with a concentration $c_{S'}$ of BTrMe but when the concentration, c_F, of pentyl TEA was superimposed the concentration of BTrMe was increased to $c_{S''}$, $c_{S''}$ being equal to $c_{S'}(1 + c_F K_F)$, $K_F = 3.6 \times 10^4$ M^{-1}. In this way the occupancy of BTrMe in equilibrium with the fast should be the same as before, although the total occupancy is increased.

Therefore if interaction were rate-limiting, although the fast antagonist would equilibrate very quickly with the receptors not occupied by the slow, the increased concentration of BTrMe would prevent it being 'displaced'. As BTrMe's occupancy is unaltered the degree of antagonism would change from one level to the next with no intermediate transitional stage.

If access were rate-limiting the concentration of fast antagonist in the proximity of the receptors would rise quickly to its equilibrium level and so the fast would equilibrate quickly with the receptors not occupied by

Table 4 The interaction between benziloyltropine methyliodide (BTrMe) and *n*-pentyl triethyl ammonium iodide (pentyl TEA)

n	*Initial concentration of BTrMe ($c_{S'}$)* (M)	*Concentration of superimposed pentyl TEA (c_F)* (M)	**Size of transitional stage*	*Observed dose-ratio* (±s.e. mean)	*Calculated dose-ratio* $\left[\frac{1 + c_{S''}K_S + c_F K_F}{1 + c_{S'}K_S}\right] = (1 + c_F K_F)$
1	2×10^{-9}	1×10^{-4}	1.1	4.7	4.6
1	2×10^{-9}	4×10^{-4}	1.2	17.9 ± 5.1	15.4
1	4×10^{-9}		1.4		
2	1×10^{-9}	8×10^{-4}	1.5, 1.4	39.4 ± 3.2	29.8
1	2×10^{-9}		2.5		
2	4×10^{-9}		2.6, 2.8		
3	1×10^{-9}	16×10^{-4}	1.7, 3.1, 2.0	55.3 ± 87.3	58.6
2	2×10^{-9}		2.2, 2.1		
2	4×10^{-9}		3.1, 1.7		

* The ratio of the equilibrium dose-ratio to the dose-ratio corresponding to the first response after the changeover gives an estimate of the size of the transitional stage. If interaction were rate-limiting the degree of antagonism would change from one level to the next with no transitional stage (i.e. 'size' = 1).

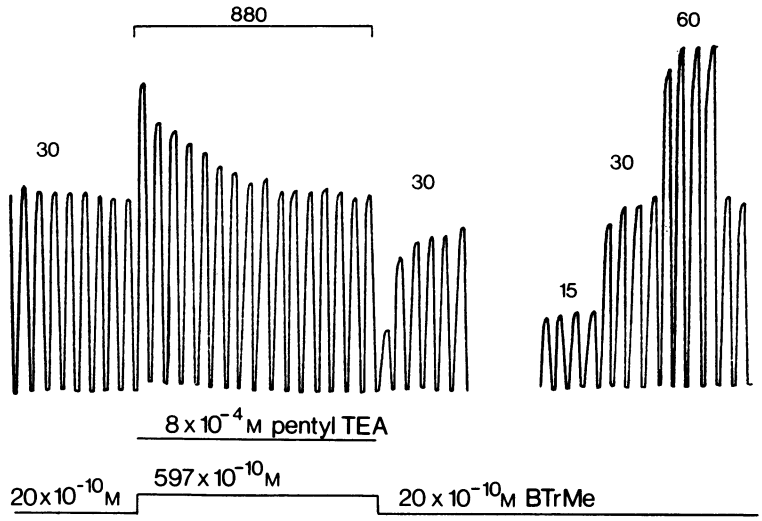

Figure 7 The interaction between benziloyltropine methyliodide (BTrMe) and *n*-pentyl triethyl ammonium iodide (pentyl TEA), the concentration of BTrMe being adjusted as shown. There is a gap in the trace of 57 minutes. The paper drive to the potentiometric recorder was on from just before the agonist was added to just after washout. The concentrations of agonist used are indicated above the responses — M x 10^7. If BTrMe's interaction with the receptors were rate-limiting, on superimposing pentyl TEA the degree of antagonism would change from one level to the next with no transitional stage.

the slow antagonist. The concentration of slow in the proximity of the receptors would, however, only rise slowly to its new level and so, because of this lag, the fast antagonist would initially 'displace' the slow antagonist from the receptors, the slow antagonist's occupancy being subsequently restored as its concentration in the proximity of the receptors rises to its new level.

Results (III)

When the concentration of fast antagonist was sufficiently large a transitional stage was observed (Figure 7, Table 4). This stage is consistent with that expected if access were rate-limiting. The extent of the initial displacement increased with the concentration of pentyl TEA as would be expected from Figure 4. Subsequently BTrMe's occupancy was slowly restored to a level consistent with that calculated taking K_F as 3.6×10^4 M^{-1}. In view of the very fast rate of onset of pentyl TEA it is unlikely that the transitional stage reflects the kinetics of this antagonist. Also the dose-response curve appeared to have shifted in a parallel manner following the superimposition of the fast antagonist and so there is no reason to believe that the agonist exposure time was insufficient for an equilibrium to be established.

IV The effect of experimental method on the kinetics of antagonism

The results of kinetic studies by different groups of workers show large differences. For instance Paton (1961), using intact pieces of ileum, found that on washout atropine's occupancy declined with a half time of about 40 min whereas Paton & Rang (1965), using muscle strips, observed half times of about 7 min although the equilibrium constants were about the same. It was therefore suggested that the observed kinetics varied with the preparation used. In addition the studies of Beraldo & Rocha e Silva (1949) and of Paton & Rothschild (1965) suggest that the kinetics of antagonism are influenced by the composition of the bathing fluid. The effect of experimental method on the kinetics of antagonism was therefore investigated in order to determine whether the discrepancies between the observed kinetics and the interaction-limited model were associated with the method used.

Results (IV)

The kinetics of BTrMe was examined using muscle strips and an isometric transducer instead of intact pieces of ileum and an isotonic lever. Both onset and

offset (Table 5) were slower than those obtained with intact pieces of ileum (Table 1), and the rate of offset did not appear to depend on the concentration. Nevertheless interaction cannot be rate-limiting as the values of τ_{off} are again considerably larger than predicted from the rate of offset on superimposition of high concentrations of pentyl TEA.

Since BTrMe leaves the receptors slowly the agonist may fail to achieve equilibrium with the receptors. Such a failure would produce a non-parallel shift in the log dose-response curve with a depression of the maximum response. Also the speed of contraction is sometimes reduced. Experiments were performed to determine the minimum concentration of BTrMe at which such effects occurred. With the isotonically loaded intact ileum this was found to be about 2×10^{-8} M which is comfortably above the concentration used in the experiments recorded in Table 1. With isometric muscle strips the effects occurred with much lower concentrations of BTrMe, down to 5×10^{-9} M, just above the concentrations used in the experiments summarized in Table 5. However, in the experiments of Table 5 there was no indication of failure of the agonist to achieve equilibrium and so it seems unlikely that such an effect could be responsible for the difference between these results and those of Table 1. The difference might be a consequence of varying degrees of damage during the preparation of the tissue perhaps causing changes in the ionic content of the tissue.

The kinetics of lachesine was also investigated under different conditions. In each experiment two pieces of ileum from the same animal were used to follow the onset and offset of action of 2×10^{-8} M lachesine and its rate of offset on superimposition of 15×10^{-4} M pentyl TEA. The four combinations of lever (isotonic or isometric) and preparation (intact pieces of ileum or muscle strips) were investigated first in Tyrode solution, the order of the experiments being determined randomly. Subsequently the kinetics of lachesine were investigated in Krebs solution. In none of these experiments (Table 6) was the kinetics of onset and offset as fast as those predicted from the rate of offset on superimposition of a fast antagonist. It is therefore unlikely that the discrepancies between the observed kinetics and the interaction-limited model are associated with the bathing medium, preparation or recording method used. Also the discrepancy

Table 5 The rates of onset and offset of benziloyltropine methyliodide (BTrMe) in muscle strips with the responses recorded isometrically. (Values are mean ± s.e. mean).

n	*Concentration of BTrMe* (c_S) *(M)*	*Time constant for onset* (τ_{on}) *(min)*	*Time constant for offset* (τ_{off}) *(min)*	τ_{off}/τ_{on}	*Equilibrium dose-ratio* (DR_∞)
3	1×10^{-9}	33 ± 12	578 ± 177	21 ± 8	25 ± 8
6	2×10^{-9}	20 ± 3	509 ± 60	27 ± 5	38 ± 5
4	4×10^{-9}	13 ± 4	502 ± 69	47 ± 9	77 ± 15

Table 6 The kinetics of lachesine (2×10^{-8} M) using 6 combinations of preparation (intact pieces of ileum or muscle strips), bathing medium (Krebs or Tyrode) and lever (isotonic or isometric). (Values are mean ± s.e. mean).

n	*Ileum prepn*	*Bathing medium*	*Lever*	τ_{on} *(min)*	$^1\tau_{off}$ *(min)*	$^2\tau_{off}\frac{1+c_SK_S+c_FK_F}{1+c_SK_S}$ *(min)*	DR_S
4	Intact	Tyrode	Isometric	4.0 ± 0.6	40.4 ± 9.7	7.8 ± 1.1	15.7 ± 0.7
4	Strips	Tyrode	Isometric	6.6 ± 2.8	36.8 ± 12.9	7.3 ± 2.5	15.0 ± 1.0
4	Intact	Tyrode	Isotonic	4.0 ± 0.3	32.4 ± 7.8	5.6 ± 1.1	15.6 ± 1.9
4	Strips	Tyrode	Isotonic	7.6 ± 1.9	33.9 ± 6.4	4.3 ± 1.0	17.8 ± 0.9
4	Intact	Krebs	Isotonic	3.7 ± 0.3	18.5 ± 4.5	5.9 ± 1.0	12.6 ± 1.0
4	Strips	Krebs	Isotonic	2.8 ± 0.3	23.0 ± 3.4	4.7 ± 0.3	15.5 ± 0.6

$^1\tau_{off}$ was calculated from the rate of offset of lachesine on washout; $^2\tau_{off}$ was calculated from the rate of offset on superimposition of pentyl TEA. If interaction were rate-limiting, $^1\tau_{off}$ and $^2\tau_{off}(1 + c_SK_S + c_FK_F)/(1 + c_FK_F)$ would both be equal to $1/k_{-1}$ and so would themselves be equal.

between the kinetics of atropine when measured by Paton (1961) and when measured by Paton & Rang (1965) cannot be due to the former using intact pieces of ileum and the latter using muscle strips. Other workers have not found the kinetics of atropine to be faster when muscle strips are used: Furchgott (quoted by Paton, 1967b) using intact pieces of ileum measured offset half times nearer 10 min than 40 whereas Thron & Waud (1968, Figure 8) using muscle strips observed rates of offset considerably slower than 10 minutes.

Discussion

In these experiments the kinetics of onset and offset of competitive antagonists do not appear to be compatible with the simple mass action model of Paton (1961). This is consistent with kinetic measurements on many other tissues (Furchgott, 1955; Paton, 1967a; Waud, 1967; Thron & Waud, 1968). Therefore either access of the antagonist to the receptors is rate-limiting or, if interaction with the receptors is rate-limiting, some alternative model is required to describe the kinetics of antagonism. If access is rate-limiting, the rate of offset of a slow antagonist on superimposing a sufficiently high concentration of a fast antagonist may allow the determination of k_{-1} and if this is so the results of Figure 4 indicate that BTrMe dissociates slowly from the muscarinic receptors with a time constant of about 20 minutes. This would largely account for its high potency which in turn would account for the extremely slow onset of its action. Unfortunately without a clear idea of the nature of the access limitation the possibility that some other access limitation is operating when high doses of the fast antagonist are superimposed cannot be ruled out. Similarly if interaction with the receptors is rate-limiting, it would not seem reasonable to calculate antagonist receptor rate constants from the observed kinetics without a plausible new interaction model. The problem is therefore can an interaction or access-limited model be derived which adequately describes the observed kinetics?

The variability of kinetic measurements would seem more probable in an access-limited than in an interaction-limited situation. Not only do the kinetic measurements of a single group show considerable variation as was noted by Thron & Waud (1968) and also in this study, but also there are discrepancies between the observations of different groups of workers. The behaviour of atropine has already been mentioned. Similarly in this study lachesine's occupancy declined on washout with a time constant greater than 20 min whereas Paton & Rang (1965) observed rates corresponding to time constants of about 4 minutes.

If then the kinetics of antagonism reflect the rate at which the concentration of drug in the proximity of the receptors approaches that in the bathing medium, what determines this rate? For potent slow antagonists such as atropine and BTrMe this cannot be limited by diffusion alone as the difference between their speed and that of a weak antagonist such as pentyl TEA cannot be explained in terms of their molecular weight. Also, using the method of analysis described by Thron (1972), the rates of change of dose-ratio were not found to be consistent with simple diffusion or any other situation describable by a linear multicompartmental model.

If, however, the amount of drug taken up by the receptors is not quantitatively negligible, as the binding studies of Paton & Rang (1965) suggest, this would introduce non-linearity into the system and would also explain why only potent antagonists exhibit very slow onset and offset of action. Because of their potency such antagonists are tested in low concentrations and so the amount of drug taken up by the receptors will be appreciable compared with the amount in the volume with which the receptors equilibrate. This will delay the attainment of an equilibrium in the proximity of the receptors and so will slow the onset and offset of antagonism. With a weak antagonist a much higher concentration has to be used to produce the same degree of receptor block. Therefore the amount of drug taken up is small compared with that in the proximity of the receptors and so uptake will have a negligible effect on the approach to equilibrium. The apparent acceleration seen in Figure 4 can also be explained in this way.

Thron & Waud (1968) used a very similar argument to explain their observation that pretreatment with an irreversible antagonist increased the rate of onset and offset of a slow antagonist. They considered that the kinetics of antagonism may reflect the rate at which the antagonist penetrates through the extracellular space by diffusion, this being slowed by uptake onto the receptors on the more peripheral cells. Alternatively though, the above effects might be due to there being some sort of barrier between the bulk of the extracellular space and the receptors. The antagonist might then equilibrate relatively quickly with the bulk of the extracellular space and the kinetics of antagonism reflect the rate at which the concentration of drug to which the receptors are exposed approaches that in the bulk of the extracellular space, this being slowed by uptake onto the receptors.

The possibility of a barrier was discarded by Rang (1966) because he felt that, for guinea-pig isolated ileum, there was no physical structure which could act as such a barrier. However, electron micrographs show that between the trilaminar membrane and the bulk of the extracellular space is a layer called the basement membrane. Greengard & Straub (1958)

suggested that this layer might be responsible for the apparently slow rates of diffusion of ions that they observed. If the diffusion of ions is affected by this layer might not the diffusion of drugs be also? Colquhoun & Ritchie (1972), in fact, mentioned the possibility that the kinetics of tetrodotoxin might be affected by this layer.

A barrier would more readily explain the effect of ionic environment on the kinetics of antagonists seen here and by Paton & Rothschild (1965) than a change in the rate of penetration. It would also explain why the kinetics are not faster when strips are used rather than intact pieces of ileum, although this could alternatively be due to the folding of the strips in the organ bath or to the agonist acting by setting up a pacemaker as was considered by Thron & Waud (1968). This would also predict that the kinetics of binding to tissue homogenates might not be appreciably faster than the kinetics of antagonism even if the kinetics of antagonism were access-limited. However, the barrier would have to have rather extraordinary powers of discrimination if it were to explain the transition stage seen in Figure 7 and the finding that in certain conditions the offset of BTrMe is faster following equilibration with higher concentrations. If the basement membrane is considered to be the most likely candidate for this barrier it may be possible to derive a plausible new kinetic model when more information is available concerning if and how such a membrane behaves in this way.

Alternatively if interaction is rate-limiting, a pacemaker mechanism of agonist action might seem to be necessary to explain why uptake onto the receptors does not cause appreciable slowing of antagonisms. If estimates of the binding capacity (M) of the muscarinic receptors are considered together with estimates of the size of the extracellular space (largest estimate of V), for slow antagonists the value of MK/V (Colquhoun & Ritchie, 1972) would seem to be sufficiently large (> 400) for appreciable slowing to be expected. However, if agonists act by setting up a pacemaker in the more peripheral cells, the kinetics of antagonism would reflect equilibration of the antagonist with these cells and not the rate of penetration through the tissue however slow this might be. This would predict, as Thron & Waud (1968) pointed out, a difference between the kinetics of antagonism and the kinetics of binding to intact tissues as seen by Paton & Rang (1965).

Before a new interaction model can be derived, modification of the simple mass action model would seem to be necessary to accommodate the change in the apparent affinity of pentyl TEA with concentration and also its combined dose-ratios with BTrMe and lachesine (see Methods section). Another anomalous observation was also made; in 39 out of 40 experiments the dose-response curve in the presence of desoxylachesine was slightly steeper than in its absence, this increase being too small to affect appreciably the calculation of dose-ratio. Both these observations could be attributed to the receptor existing in different forms.

In addition a plausible new interaction model would have to explain how the kinetics of antagonism can change without changing the apparent affinity at equilibrium. For instance pretreatment with an irreversible antagonist (Thron & Waud, 1968) and superimposing a high concentration of a fast antagonist, accelerate the kinetics of offset of a slow antagonist without altering the apparent equilibrium position. Similarly the variation in kinetic measurements already discussed does not seem to be associated with changes in the apparent affinity at equilibrium. There is also some evidence that ion levels can influence the observed kinetics of antagonism without changing the apparent affinity at equilibrium (Beraldo & Rocha e Silva, 1949; Paton & Rothschild, 1965), but in this study, although the bathing medium does appear to influence slightly the observed kinetics of lachesine (Table 6), the equilibrium dose-ratios were also slightly affected ($0.01 < P < 0.05$: 2 factor analysis of variance — interaction not significant). Differences in rates in these experiments may therefore be partly associated with changes in the apparent affinity constant.

In conclusion, it may be possible to derive a kinetic model based on a modification of the simple mass action model which is reasonably consistent with the observed kinetics and is also consistent with the available information concerning the binding of muscarinic agonists and antagonists to the receptors. However, without such a model or a plausible access model it would seem impossible at this time to decide whether access or interaction normally limits the kinetics of potent competitive antagonists on isolated pieces of guinea-pig ileum, or even whether both factors contribute to the observed kinetics of antagonism.

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