An Alkylating Derivative of Benzilylcholine with Specific and Long-Lasting Parasympatholytic Activity

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SUMMARY

Benzilylcholine mustard (*N*-2-chloroethyl-*N*-methyl 2-aminoethyl benzilate), a 2-haloalkylamine which cyclizes in solution to form an ethyleniminium derivative structurally very similar to benzilylcholine, was found to be a potent antagonist of the muscarinic action of acetylcholine. It differed from benzilylcholine in showing a much greater persistence of action, which was attributed to the alkylating activity of the ethyleniminium ion. Its action was highly specific, antagonism of the action of histamine and noradrenaline on smooth muscle requiring about 1000 times the concentration needed to block acetylcholine. It did not block ganglionic or neuromuscular transmission, but inhibited parasympathetic effects as well as the muscarinic actions of exogenous acetylcholine.

Blocking activity was confined to the ethyleniminium ion: neither the 2-chloroethylamine nor the alcoholic hydrolysis product was active.

In a study of the rate constants relating to the interaction of benzilylcholine and benzilylcholine mustard with acetylcholine receptors in guinea pig intestinal muscle, it was found that the association rate constant was similar for the two compounds, but that dissociation following alkylation by benzilylcholine mustard occurred at only $\frac{1}{300}$ of the rate at which benzilylcholine dissociates. The complex initially formed by benzilylcholine mustard was a reversible one, but alkylation occurred rapidly, the rate of alkylation being considerably greater than that of dissociation of the reversible complex.

INTRODUCTION

The series of catecholamine antagonists characterized by the possession of the 2-haloethylamine group has been studied very extensively and has been shown by Nickerson, Graham and their co-workers (1–3) to depend for its action on the formation of a reactive quaternary ethyleniminium derivative. The strikingly persistent blocking action of many of these compounds is attributed to the alkylation of a nucleophilic group associated with the catecholamine α-receptor by the ethyleniminium ion either in its cyclic form or in an open carbonium form (4).

A more obvious site of attack for quaternary ethyleniminium compounds than the catecholamine α-receptor would seem to be the muscarinic acetylcholine receptor, for which numerous quaternary stimulants and antagonists are known. This paper concerns the investigation of a 2-chloroethylamine of which the ethyleniminium derivative is a close structural analog of the potent atropine-like compound benzilylcholine (5). The compound studied was *N*-2-chloroethyl-*N*-methyl-2-aminoethyl benzilate, which we have called benzilylcholine mustard (BCM) on account of its relationship to the nitrogen mustards. Its structure is shown in Fig. 1, which also illustrates the assumed reactions by which the ethyleni-

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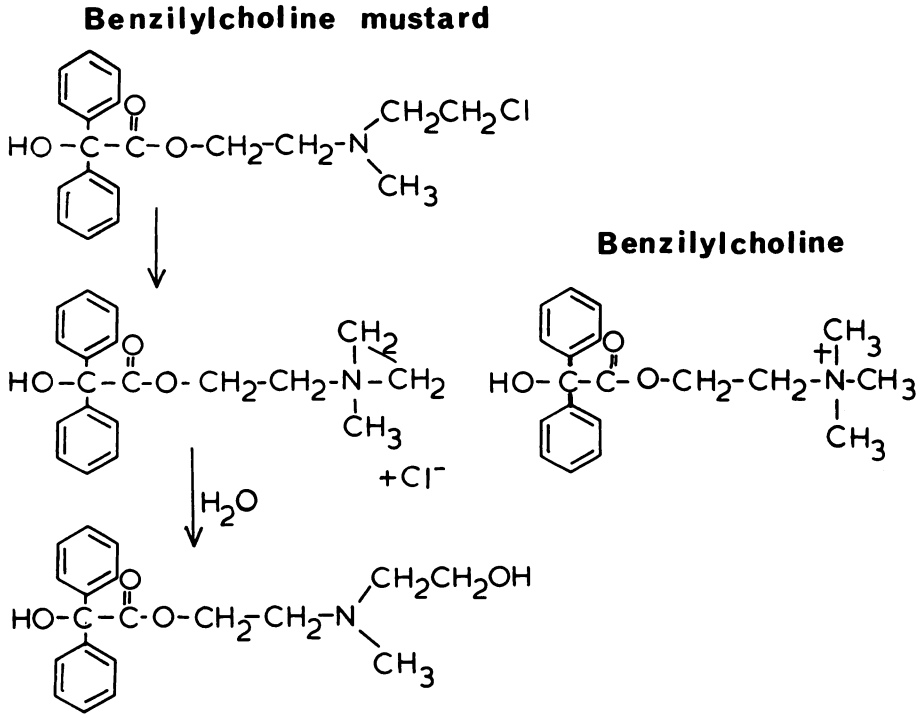

FIG. 1. *Structural formula of benzilylcholine mustard, showing similarity of its ethyleniminium derivative to benzilylcholine*

minium derivative is formed and subsequently hydrolyzed.

Recent studies (6, 7) in which the persistent blocking action of such drugs as dibenamine on acetylcholine and histamine receptors has been used in order to estimate the affinity of various agonists for receptors, have yielded results somewhat at odds with those obtained by other methods (8, 9). There is clearly doubt as to whether these highly reactive and nonspecific antagonists act purely by blocking muscarinic receptors in the concentrations at which they are used (10^{-6} to 10^{-5} M). A specific and potent alkylating agent for acetylcholine receptors would be much more suitable for studying the affinity of muscarinic agonists for receptors.

The present paper is concerned with the chemical transformations and pharmacologic activity of BCM, which has been found to possess the properties of high potency, specificity, and persistence of action likely to render it useful in studying the properties of muscarinic receptors.

METHODS

Synthesis of BCM. A mixture of benzilic acid (0.1 mole) and bis-2-chloroethylmethylamine (0.1 mole) (10) in dry isopropanol (400 ml) was refluxed, with careful exclusion of water, for 24 hr. The solvent was removed under reduced pressure, the residue was shaken with 2 N hydrochloric acid (30 ml), and the acid-insoluble material was removed by extraction twice with ether. The clear aqueous solution was then cooled in ice, made alkaline with 6 N sodium hydroxide, and extracted twice with cold ether. The ethereal extract was dried with magnesium sulfate for 15 min, filtered, and then saturated with dry hydrogen chloride. The oil that separated crystallized on standing overnight. The product was recrystallized from acetone, the solution being kept in the refrigerator overnight before filtering. After 4 recrystallizations an analytically pure sample was obtained, which gave only a single spot on high voltage electrophoresis [formate/acetate buf-

fer, pH 2.0; 100 v/cm; developed with Dragendorff's reagent (11)].

Yield 3.0 g (8%); m.p. 156				
Analysis	C	H	N	Cl
Found (%)	59.5	5.92	3.72	18.3
$C_{19}H_{22}O_3Cl_2$ requires (%)	59.4	5.99	3.65	18.5

Measurement of formation and hydrolysis of the ethyleniminium derivative. The method used was a modification of the procedure described by Chapman and James (12). The greater water solubility of BCM made it possible to measure ethyleniminium concentrations in buffered aqueous solution, which was thought preferable to the ethanol–water or acetone–water mixture used in earlier studies.

The reaction mixture contained BCM (0.8 mM) and phosphate buffer (10 mM). Most of the measurements were made at pH 7.3. A few measurements at higher pH were made in the presence of 10 mM glycine:NaOH buffer. The solution was kept at constant temperature. Aliquots, 5 ml, were removed at intervals, and the reaction was stopped by adding 0.2 N acetic acid (1 ml). Then 1.0 ml of 10 mM sodium thiosulfate was added and allowed to react with the ethyleniminium ion for 20 min at room temperature. The residual thiosulfate was estimated by titration with 4 mM iodine solution. The reaction of the ethyleniminium ion with thiosulfate was complete within 10 min, and repeatable results were obtained when titrations were done between 10 and 20 min after adding thiosulfate.

Chloride ion released during cyclization was measured by assaying 1.0-ml aliquots by means of an electrometric chloridometer (Buchler-Cotlove Instruments).

In an experiment in which mydriatic activity in mice was correlated with ethyleniminium concentration during incubation of a solution of BCM at pH 7.3, 10-ml aliquots of the reaction mixture were shaken with chloroform (2×10 ml) in order to extract unchanged 2-chloroethylamine. The aqueous phase was then diluted (using 0.9% NaCl solution previously shaken with chloroform) and used for the mouse assay. This extraction was necessary as remaining 2-chloroethylamine would have cyclized quickly *in vivo,* and this would have interfered with the assay.

Studies on isolated tissues. Studies of the time course and specificity of action of BCM were made on isolated strips of longitudinal muscle from guinea pig ileum (13) and on guinea pig vas deferens. The tissues were suspended in 10 ml Krebs solution at 37°, bubbled with 95% oxygen:5% carbon dioxide. The Krebs solution was of the following composition (mM): NaCl 113, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5, dextrose 11.5. In the experiments on intestinal muscle, hexamethonium bromide (7×10^{-5} M) was added to the Krebs solution in order to prevent possible ganglionic stimulation by high concentrations of agonists.

Contractions were recorded isometrically, using a valve transducer (RCA 5734) and a pen recorder.

The stimulant drug used in most of the studies on intestinal muscle was methylfurmethide (14). This is an acetylcholine-like agonist of high potency, and was used because it is not hydrolyzable, either spontaneously or enzymically, and seems to have rather less ganglion-stimulating activity than carbachol. In its sensitivity to muscarinic blocking agents, methylfurmethide is indistinguishable from acetylcholine.

The procedure used for measuring the kinetics of action of antagonists was the same as that used by Paton and Rang (8).

Measurement of mydriatic activity in mice. The method of Ing *et al.* (5) was used, with the modification that drugs were injected intravenously in a volume of 0.2 ml. This was found to give much more consistent results than intraperitoneal injection. Pupil diameter was measured before, and at various times after, the drug by observation in bright light under a dissecting microscope (magnification ×10) fitted with a measuring graticule.

Albino male mice (20–25 g of a uniform, Caesarean-derived strain were used. Groups of 6–10 mice were used for each measurement, and each mouse was used only once. Drug doses were not adjusted

according to individual body weights, but are expressed in relation to the mean body weight measured for each batch of mice.

Cat experiments. A few experiments were done on cats anaesthetized first with ether, then with chloralose (80 mg/kg i.v.). Conventional methods were used for recording blood pressure, heart rate, contractions of the nictitating membrane in response to preganglionic stimulation, and the indirectly elicited twitch of the tibialis anterior muscle.

Estimation of anticholinesterase activity. The colorimetric method described by Katsh (15) was used. The cholinesterase preparation used consisted of dog caudate nucleus ground up in Krebs solution (100 mg fresh tissue per milliliter). The equivalent of 1 mg tissue was used in each incubation flask, and approximately 70% of the acetylcholine was hydrolyzed in 30 min.

Analog computation. To analyze the theoretical kinetics of reaction of an alkylating agent with receptors, a PACE TR-20R computer (Electronic Associates Ltd) was used.

RESULTS

Chemical Transformations of BCM in Solution

Cyclization to ethyleniminium can occur only if the 2-haloethylamine is in its nonionized form. It was therefore important to determine the effect of pH on the rate of cyclization. Figure 2 shows the release of chloride ion at 20° by solutions containing 1 mM BCM at different pH's. When buffers of pH greater than 6 were added to the BCM dissolved in water, a faint precipitate of free base appeared, which redissolved within 2 min. This did not occur if the BCM concentration was 0.8 mM or less.

Figure 2 shows that the rate of chloride release increased with pH up to pH 7.3, but did not increase beyond this. Ionization of the BCM must therefore be negligible at pH 7.3, and the curves in Fig. 2 suggest that its pK_a must be approximately 6, a value consistent with measurements on other tertiary 2-chloroethylamines (16). The pH of an unbuffered solution containing 1 mM BCM was 4.0, and the release of chloride amounted to only about 5% in 24 hr.

Studies of the formation and hydrolysis of ethyleniminium were made using 0.8 mM BCM at pH 7.3. The results are shown in Fig. 3. At room temperature the maximum conversion to ethyleniminium ion (82%) was reached in 1 hr. Hydrolysis at this temperature was very slow, the ethyleniminium ion concentration falling by only 2% in 2½ hr. Chloride release followed an approximately exponential time course, with a half-time of 14 minutes. At 37° the half-time for chloride release was 2.3 min, and the peak ethyleniminium level of 83% (the same as at room temperature) was attained in 10 min. Disappearance of the ethyleniminium ion was fairly rapid at 37°, 50% being hydrolyzed in about 1½ hr.

At 23°, the maximum conversion of ethyleniminium ion was only 82% although the rate of hydrolysis of ethyleniminium was negligible. The remaining 18% of the starting material must thus have been converted, by elimination of halide, to a species which did not react with thiosulfate. A similar phenomenon was seen by Harvey and Nickerson (17) in their study of the rearrangements of dibenamine and phenoxybenzamine in solution, and they postulated the formation of a piperazinium dimer. A similar compound could be formed by BCM. The rate constant for chloride release tended to fall slightly with time, which would suggest that part of the release occurred in a bimolecular reaction, but we have not investigated it further, as this side product did not appear to contribute to the pharmacologic activity (see later results).

A single experiment was carried out under experimental conditions similar to those used by Chapman and James (12). The reaction mixture comprised: BCM 60 μmoles, NaOH 100 μmoles, water 2.1 ml, acetone 4.9 ml. At 30° the maximum conversion to ethyleniminium ion under these conditions was less than 30%, and was reached in about 1 hr, when chloride release was only 50% complete. Subsequent hydrolysis of the

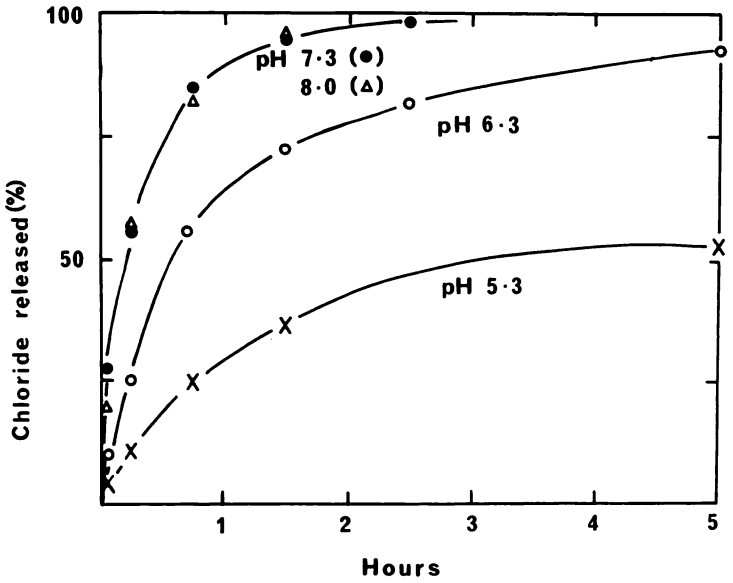

FIG. 2. *Release of chloride ion from 1 mM BCM in aqueous solution at 20°, buffered with 10 mM phosphate buffer (pH 5.3–7.3) or 10 mM glycine—NaOH buffer (pH 8.0)*

Ordinate shows chloride released as a percentage of the maximum of 1 equivalent per mole BCM.

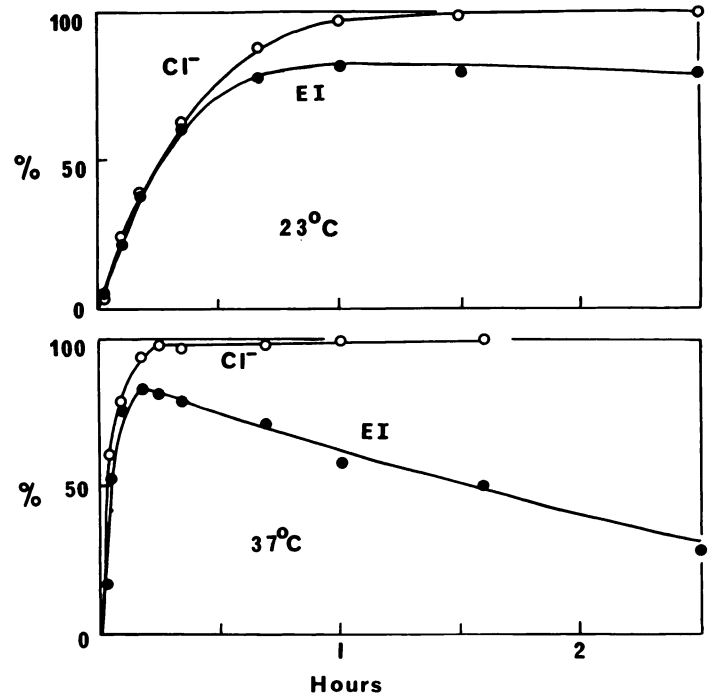

FIG. 3. *Chloride and ethyleniminium ion formation in solutions containing 0.8 mM BCM in 10 mM phosphate buffer, pH 7.3*

Ordinate shows chloride ion released and ethyleniminium ion (EI) formed as a percentage of the maximum of 1 equivalent per mole BCM.

ethyleniminium ion appeared to be rather faster than in aqueous solution at 37°.

By analogy with the catecholamine antagonists it seemed likely that alkylating activity would be confined to the ethyleniminium derivative, and later results confirmed this, so in the pharmacologic studies, a solution containing 0.8 mM BCM at pH 7.3 was kept for 1–1½ hr at room temperature before use. This solution was then stored in a refrigerator for up to 6 hours, and the ethyleniminium concentration was assumed to be 80% of the starting concentration. Except where otherwise stated, the BCM concentrations quoted refer to this calculated ethyleniminium concentration.

Pharmacologic Studies

BCM was found to be a potent and extremely persistent antagonist at muscarinic acetylcholine receptors. A convenient measure of drug antagonism is the agonist dose ratio (DR), i.e., the ratio by which the agonist concentration must be increased in order to produce a standard contraction during the action of an antagonist. Paton (18) has used the following relationship to obtain the fraction of receptors (p) occluded by the antagonist, from DR:

$$p = (DR - 1)/DR$$

The validity of this equation depends on the antagonism taking the form of a parallel shift to the right of the agonist log-dose response curve, which is often regarded as indicating "competitive" antagonism, but can occur (9) with persistent antagonists if the agonist occupancy producing the measured effect is very low. In the present experiments with acetylcholine or methylfurmethide acting on guinea pig intestinal muscle, the parallel shift of the log-dose response curve produced by BCM was maintained up to a dose ratio of about 50. At higher levels of antagonism, some flattening was apparent.

In the kinetic studies to be described, all the measurements were made at dose ratios lower than 50, so the above equation for antagonist occupancy was valid even though the antagonism could not be regarded as competitive.

Figure 4 shows the effect of BCM on the responsiveness of a strip of intestinal muscle to methylfurmethide. After exposure of the tissue to BCM (3.2×10^{-9} M) for 9 min, the response to 4×10^{-8} M methylfurmethide was about equal to that given by 8×10^{-9} M methylfurmethide before BCM had been applied (i.e., a dose ratio of 5 was achieved, corresponding to 80% occupancy). Recovery of sensitivity after washing out the BCM was very slow, occupancy having declined only to 60% after 4 hr. Recovery from benzilylcholine antagonism was complete within about 10 min of washing it out (see Fig. 5).

When tested against histamine contractions of guinea pig intestinal muscle, BCM was very much less active than against acetylcholine or methylfurmethide, the blocking concentration being 500–1000 times greater. At BCM concentrations below 4×10^{-7} M, no effect on histamine contraction was usually discernible. In guinea pig vas deferens preparations, acetylcholine contractions were as sensitive to BCM as

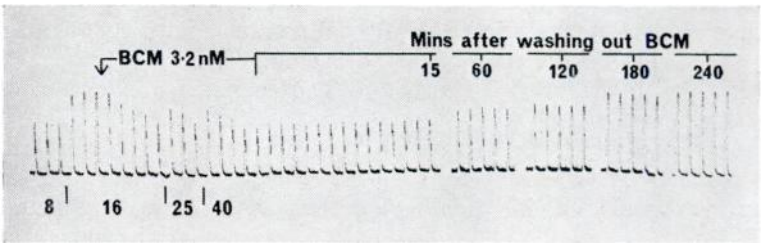

FIG. 4. *Tracing of isometric contractions of isolated strip of longitudinal muscle of guinea pig ileum at 37°, in response to methylfurmethide injected every 60 sec and washed out after 15 sec*

Figures beneath tracing show bath concentrations (nM) of methylfurmethide. BCM was present for 12 min and was then washed out.

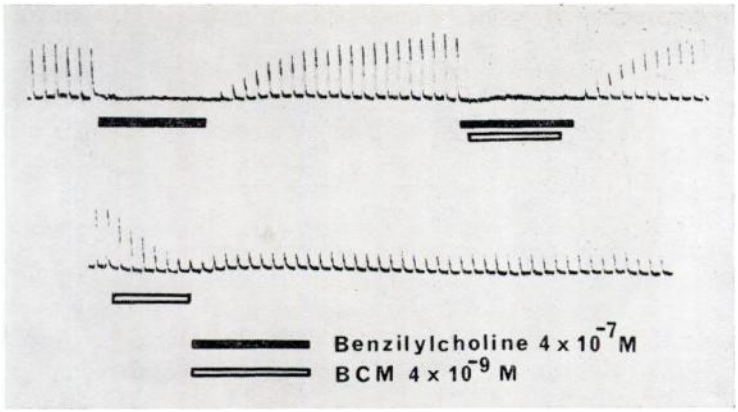

FIG. 5. *Tracing as in Fig. 4*

Methylfurmethide dose was 2×10^{-6} M throughout. BCM added in the presence of benzilylcholine failed to produce a persistent block. The record is continuous.

in the ileum, but noradrenaline contractions were affected only by BCM concentrations of 10^{-6} M or more. When antagonism to histamine or noradrenaline by BCM was established, no appreciable recovery occurred within 1 hr of washing out the antagonist.

In anesthetized cats, BCM (0.6 μmole/kg intravenously) antagonized the depressor action of acetylcholine to a dose ratio of about 100, and abolished the effect of stimulating the R. vagus nerve (supramaximal 1 msec stimuli at 10/sec for 10 sec) on the heart rate. This dose also caused maximal pupillary dilatation. At 20 times this dose (12 μmole/kg) BCM did not block the twitch of tibialis anterior, nor the response of the nictitating membrane to preganglionic nerve stimulation (supramaximal 1 msec stimuli at 10/sec for 30 sec), nor the pressor effect of injected noradrenaline.

The cholinesterase activity of homogenized dog caudate nucleus was unaffected by 10^{-4} M BCM, and the BCM did not appear to be appreciably hydrolyzed by the enzyme.

A further test of the specificity of action of BCM was made by measuring the protecting action of benzilylcholine against BCM block in intestinal muscle. The phenomenon of receptor protection has been used by Furchgott (19) and others as a method of discriminating different types of receptor. Figure 5 shows a tracing of the responses of a strip of intestinal muscle to a fixed dose of methylfurmethide applied every 60 sec. Benzilylcholine, 2×10^{-7} M produced immediate complete block (this concentration was sufficient to block approximately 99% of the receptors), and the tissue recovered fully within about 10 min of washing out the antagonist. When, during the action of benzilylcholine, BCM (4×10^{-9} M) was added to the bath, recovery after washing out both drugs was again complete in about 10 min. BCM on its own, however, produced a persistent block. Though not shown in Fig. 5, addition of benzilylcholine after BCM had been allowed to act did not hasten recovery. Thus benzilylcholine was able to protect against, but not reverse, the action of BCM. Experiments with hyoscine gave a similar result. This finding supports the view that both drugs act at the same receptor site. The alternative explanation, that benzilylcholine reacts with BCM in solution, seems very unlikely.

The persistence of action of BCM compared with benzilylcholine was- evident also in studies of their mydriatic action in mice. Figure 6 shows the time course of action of these two compounds.

The nature of the substance responsible for the blocking action of BCM was studied by assaying the mydriatic activity of solutions of BCM at different stages in the cycle of generation and hydrolysis of the

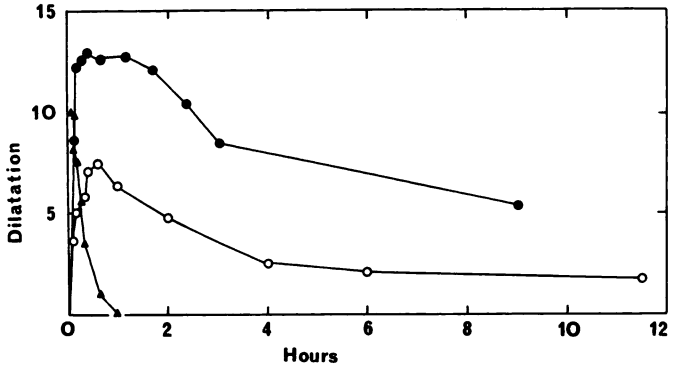

FIG. 6. *Mydriatic action of BCM and benzilylcholine in mice*

Drugs were given intravenously in 0.2 ml saline. Ordinates show the increase in pupil diameter (mean of 10 mice) measured in arbitrary units. Control injections of saline had no effect. ● BCM 0.24 μmole/kg; ○ BCM 0.12 μmole/kg; ▲ Benzilylcholine 0.38 μmole/kg.

ethyleniminium ion, shown in Fig. 3. BCM, 0.8 mM, at pH 7.3 was kept at 20° for 1 hour, aliquots being removed at intervals and immediately shaken with chloroform to remove unchanged 2-chloroethylamine. The solutions were then assayed for ethyleniminium ion concentration and for mydriatic activity in mice. After 1 hr at 20° the reaction mixture was kept at 37° to hasten hydrolysis, and further samples were taken. From each sample two dilutions were selected (one twice as great as the other) so that both produced an appreciable but submaximal mydriatic effect. Each dose of each sample was injected into 8 mice, and the pupil diameters were measured 30 min later. The results were analyzed as a series of 2 + 2 assays (20) using the sample of highest activity (that obtained after 1 hr at 20°) as a standard. The results are shown in Fig. 7. Mydriatic activity ran closely parallel to the ethyleniminium concentration. Two points on the curve show a disparity between the 5% limits of error of the mydriatic potency, and the ethyleniminium concentration. This is probably not

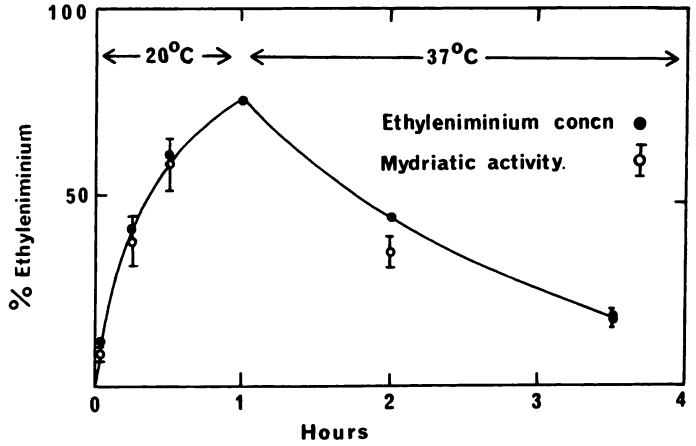

FIG. 7. *Ethyleniminium ion concentration* (●) *expressed as percentage of maximum possible (corresponding to 1 equivalent per mole BCM), and mydriatic activity in mice* (○)

The mydriatic activity is expressed in terms of the activity of the sample taken at 1 hour. Vertical bars show 5% confidence limits. The BCM solution (0.8 mM at pH 7.3) was kept at 20° for 1 hour, then at 37° to hasten hydrolysis of the ethyleniminium ion. After 18 hours at 37° neither ethyleniminium nor mydriatic activity were measurable.

significant as no estimate of the error of the ethyleniminium titration was available. After 18 hr at 37° neither ethyleniminium nor mydriatic activity was detectable, and it was concluded that the solution contained less than 2% of the mydriatic activity of the standard. These results suggest that neither the hydrolysis product nor side products of the chloride-eliminating reactions contributed appreciably to the biological activity. This accords with the findings of Graham and Lewis (2, 3) using catecholamine antagonists. This experiment did not rule out the possibility that the unchanged 2-chloroethylamine might be active, as this was extracted from the assay solutions (the 2-hydroxyethyl hydrolysis product, being a stronger base, would have remained in the aqueous phase).

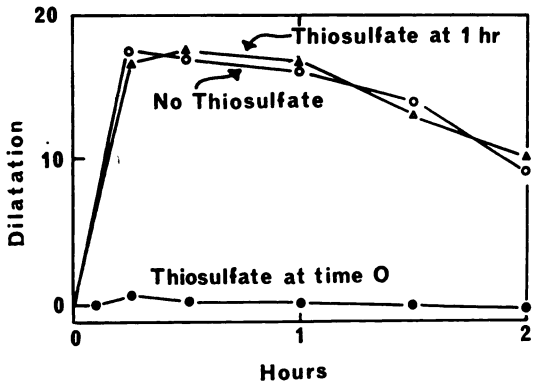

FIG. 8. *Effect of sodium thiosulfate on the mydriatic action of BCM in mice*

Open circles (○) show the effect of BCM (0.36 μmole/kg) on its own. Closed circles (●) show the effect of injecting simultaneously 4 mmole/kg sodium thiosulfate. Triangles (△) show that sodium thiosulfate (injected 1 hr after the BCM) was without effect once the mydriasis was established. Sodium thiosulfate had no effect on the mydriasis produced by benzilylcholine.

In order to detect activity in the parent 2-chloroethyl compound, the effect of simultaneous injection of sodium thiosulfate on the mydriatic activity of uncyclized BCM was investigated. It is known that thiosulfate reacts quickly with ethyleniminium ions, but not with the parent haloethylamines, and it has been shown (21, 22) to prevent but not to reverse α-receptor blockade by 2-haloethylamines. Injection of uncyclized BCM with thiosulfate should allow any pharmacologic action of the 2-chloroethylamine to appear without interference from ethyleniminium. Figure 8 shows that sodium thiosulfate injected with uncyclized BCM completely blocked its mydriatic activity, but had no effect when injected after the mydriasis had become established. The same dose of thiosulfate had no effect on the mydriasis produced by benzilylcholine.

The whole of the blocking activity of BCM thus seemed to reside in its ethyleniminium derivative. This finding was to be expected, as both the precursor and the hydrolysis product are tertiary bases, and Ing *et al.* (5) found that the tertiary derivatives of their series of choline esters in general possessed only feeble blocking activity.

Kinetics of Action of BCM

Studies made in this laboratory (8, 9, 18) support the idea that the kinetics of action of reversible muscarinic blocking agents in guinea pig intestinal muscle reflect the rate of the reaction of the antagonists with receptors, and are not seriously distorted by diffusive delays. It was therefore of interest to measure the rate constants for the reaction of the ethyleniminium derivative of BCM with the receptors, and to compare the results with those obtained with the nonalkylating analog benzilylcholine.

The sequence of reactions which we may postulate for an alkylating agent is:

$$A + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (AR)_1 \xrightarrow{k_3} (AR)_2 \xrightarrow{k_4} R + A'$$

A represents the alkylating agent, R the receptor. Complex $(AR)_1$ represents a reversible drug-receptor complex, and $(AR)_2$ the alkylated complex. The final reaction describes the slow restoration of the receptors, A′ being a modified form of the drug, probably the 2-hydroxyethylamine.

The rate constant for recovery (k_4) can be roughly estimated by means of experiments such as that shown in Fig. 4. By following the decline of the agonist dose

ratio (DR) for a number of hours after washing out the BCM, we can estimate the rate of decline of receptor occupancy.

Since recovery is a monomolecular dissociation, it would be expected to proceed exponentially, with rate constant k_4. This could not be tested experimentally because recovery was very slow, but assuming it to be so, the rate constant in six different experiments ranged from 0.04 to 0.08 hr^{-1}, with a mean value of 0.055 hr^{-1}, equivalent to a half-time for recovery of about 12 hr.

The rate equations applying to the above reaction sequence are:

$$\frac{dp_1}{dt} = k_1x(1 - p_1 - p_2) - (k_2 + k_3)p_1$$

$$\frac{dp_2}{dt} = k_3p_1 - k_4p_2$$

where p_1 and p_2 are the respective fractions of the total receptor pool occupied reversibly (AR_1) and irreversibly (AR_2), and x is the drug concentration.

Since the rate constant for recovery (k_4) was very low, it could be neglected without serious error, simplifying the solution considerably. The analytical solution of these equations is of the form:

$$p_1 + p_2 = 1 + \frac{1}{k_3(m_1 - m_2)} m_2(m_1 + k_3) \exp(m_1t) - m_1(m_2 + k_3) \exp(m_2t)$$

where m_1 and m_2 are the two roots of the quadratic equation:

$$m^2 + (k_1x + k_2 + k_3)m + k_1k_3x = 0$$

The roots m_1 and m_2 are sufficiently cumbersome that the behavior of the system was studied more easily by analog computation. Curves of total occupancy ($p_1 + p_2$) against time were plotted for various drug concentrations and various values of the rate constants k_2 and k_3. From the computed curves the general properties of the system were found to be as follows:

(a) If $k_3 \gg k_2$ total occupancy ($p_1 + p_2$) increases exponentially from zero to 1, with a rate constant $-k_1x$.

(b) If k_3 is only slightly greater than k_2 ($k_3/k_2 > 2$) total occupancy increases according to the sum of two exponential terms, but the two exponents are sufficiently close that experimental measurements might fail to show a detectable deviation from simple exponential kinetics.

Under these conditions, if the apparent rate constant were used to estimate $-k_1x$ (on the assumption that $k_3 \gg k_2$), then the calculated value of k_1 would be rather too low. The maximum error in estimating k_1 would be about 30%.

(c) If $k_3/k_2 < 2$, then the double exponential kinetics would be experimentally obvious.

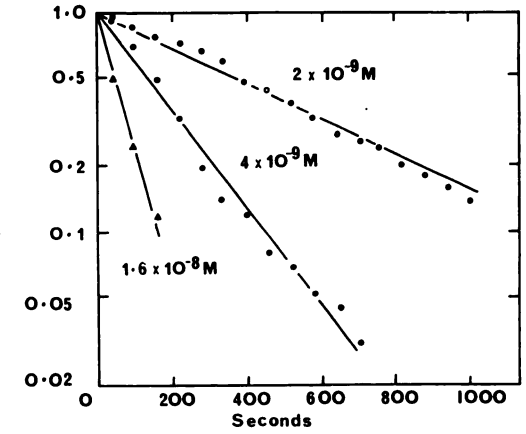

FIG. 9. *Kinetics of onset of antagonism to methylfurmethide by BCM in isolated longitudinal muscle of guinea pig ileum at 37°*

Occupancy (p) was calculated from the methylfurmethide dose ratio as described in text, and (1-p) is plotted logarithmically against time for three experiments on different preparations. Linearity indicates first-order reaction kinetics, allowing the association rate constant (k_1) to be calculated, as discussed in text.

(d) If the drug is washed out short of complete equilibration the antagonism should be partly reversible. Exposure for a brief period to a high drug concentration (when k_1x is large and k_3 rate-limiting) results in a greater degree of reversibility than if the same total occupancy is achieved by adding a lower concentration of drug for a longer time (k_1x rate-limiting).

Some typical experimental kinetics measurements at different drug concentrations are shown in Fig. 9. The value ($1 - p$) is

plotted logarithmically against time, to test whether or not occupancy develops exponentially. It can be seen that the experimental measurements did not deviate consistently from simple exponential kinetics, and there was no suggestion of a decline in the rate constant with time. This implied, as discussed above, that k_3 was considerably greater than k_2. From the rate constant describing the onset of block it was therefore legitimate to estimate the

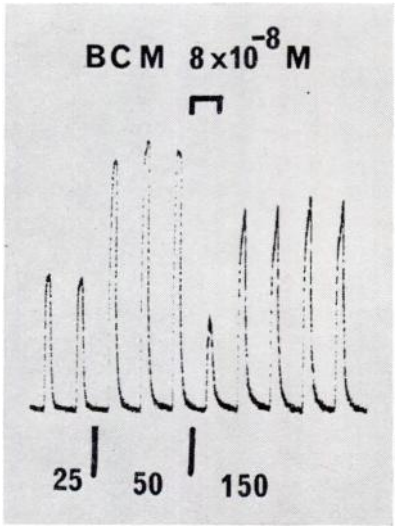

FIG. 10. *Tracing as in Fig. 4*

BCM was present for 50 seconds, and produced a block which was partly reversible. The dose ratio at the end of the exposure to BCM was 6.5 (occupancy 0.85). After the BCM had been washed out, the dose ratio fell to 3.9 (occupancy 0.74). Subsequent recovery was very slow, as in Fig. 4 tracing.

value of k_1, with maximum underestimation of about 30%. From the experiments at BCM concentrations ranging from 1.6×10^{-9} M to 6.4×10^{-8} M the mean estimate of k_1 was 1.15×10^6 M^{-1} S^{-1} (Table 1). The estimated value of k_1 did not vary systematically with BCM concentration, and this was a further indication of its validity, since this internal consistency would have been lost if the assumption that $k_3 \gg k_2$ had not been justified.

Estimation of k_3 and k_2 was more difficult. In the experiment shown in Fig. 10, a high concentration of BCM (8×10^{-8} M) was added 40 sec before methylfurmethide, and washed out after the methylfurmethide had acted for 10 sec. The antagonism produced was, as predicted, partially reversible, and the reversal was complete within 1 min of washing out the BCM. This allows us to estimate a lower limit for k_3. From the final dose ratio (3.9) achieved in Fig. 10, the irreversible occupancy, p_2, was 0.74, and the maximum total occupancy, $p_1 + p_2$, reached at the end of the exposure to BCM was 0.85. Even if $p_1 + p_2$ had risen instantaneously to 0.85 immediately BCM was added, and had remained there for 100 sec (at which time the value $p_2 = 0.74$ was established), for p_2 to reach 0.74 in this time means that 100 sec must represent at least 3 half-times for the alkylation reaction, governed by rate constant k_3. The half-time for alkylation could not therefore have exceeded 33 sec, equivalent to a lower limit for k_3 of 2.1×10^{-2} sec^{-1}. Several experiments similar to that shown in Fig. 10 were carried out, giving fairly consistent lower limit values for k_3.

It has not been possible to estimate k_2. The only approach would be by detecting a deviation from exponential kinetics in the onset of antagonism, and this has not been observed. The rate constant for alkylation, k_3, was apparently so high that dissociation of $(AR)_1$ was negligible, virtually all the reversible complexes formed proceeding to alkylation.

The rate constants k_1 and k_2 for benzilylcholine were measured using the method of Paton and Rang (8) and are included in Table 1. Measurement of the rate constant for onset of antagonism by benzilylcholine was difficult, because equilibration was usually complete within about 5 min. The value of k_1 was therefore estimated by measuring the equilibrium constant (k_2/k_1) from the occupancy attained at equilibrium, and the rate constant (k_2) for recovery after washing out the benzilylcholine, and calculating k_1 from the ratio of these two quantities.

The very slow recovery rate after block by BCM implies that the antagonism produced should be of the nonequilibrium

TABLE 1
Rate constants of BCM and benzilylcholine on guinea pig intestinal muscle at 37°

	BCM	Benzilylcholine
$k_1(M^{-1}S^{-1})$	$1.15 \times 10^6 \pm 0.13 \times 10^6$ (10)[a]	$1.69 \times 10^6 \pm 0.12 \times 10^6$ (8)
$k_2(S^{-1})$	?	$4.5 \times 10^{-3} \pm 0.2 \times 10^{-3}$ (8)
$k_2/k_1(M)$	?	$2.74 \times 10^{-9} \pm 0.11 \times 10^{-9}$ (9)
$k_3(S^{-1})$	2.1×10^{-3}	—
$k_4(S^{-1})$	$1.52 \times 10^{-5} \pm 0.17 \times 10^{-5}$ (6)	—

[a] Mean ± standard error of mean is shown, with number of measurements in parentheses.

type (23) (i.e., addition of an agonist should not cause any decline in the antagonist occupancy) and the slope of the agonist log-dose effect curve should be reduced in the presence of the antagonist. With acetylcholine and methylfurmethide, this was observed when the dose ratio produced by BCM exceeded about 50. Analysis of this flattening by means of a reciprocal plot (9) showed it to be consistent with nonequilibrium antagonism, and the equilibrium constant for both acetylcholine and methylfurmethide appeared to be about 2×10^{-6} M. In order to maintain the high dose ratios needed for these experiments, it was necessary to keep a low concentration of BCM in the bath throughout the measurements, because even the low dissociation rate of 0.06 hr^{-1} meant that recovery at high dose ratios was too fast to allow a dose-response curve to be established.

DISCUSSION

The results presented suggest strongly that BCM produces its persistent blocking action at muscarinic receptors by a mechanism closely analogous to that by which the series of 2-haloethylamines related to dibenamine act on catecholamine α-receptors. Just as with the catecholamine antagonists, we have found that the ethyleniminium ion is the active form.

Both the unchanged 2-chloroethyl compound and the alcoholic hydrolysis product were without appreciable activity. This has been found also with the α-blocking activity of the compounds studied by Graham and Karrar (24), but the antihistamine activity of some of these compounds was found to increase as hydrolysis of the ethyleniminium ion took place, owing to the greater antihistamine activity of the nonalkylating alcoholic hydrolysis product.

The kinetics of build-up and decline of ethyleniminium ion concentration differed from similar measurements made on the catecholamine antagonists in being carried out at fixed pH in aqueous solution, instead of in ethanol- or acetone-water mixtures. When the behavior of BCM in these two media was compared, it was found that the maximal conversion to ethyleniminium ion was a great deal less in acetone–water mixture than in water alone, and cyclization appeared to be slower. This may be due partly to the higher concentration used, which would favour bimolecular side reactions such as piperazinium formation and partly to the lower dielectric constant of the medium. In view of the importance attached to measurements of rates of cyclization by Belleau (25) in relation to the chemical reactions involved in combining with α-receptors, it would seem important to establish that the comparative behavior of different drugs does not depend on the solvent system used.

BCM is highly specific in its antagonistic action, having very much less action against histamine, catecholamines, and the nicotinic actions of acetylcholine than against the muscarinic receptors. This, together with the fact that benzilylcholine and hyoscine protect against BCM block, and the close chemical resemblance of its ethyleniminium derivative to benzilylcholine, leave little doubt that BCM combines specifically with muscarinic receptors.

It is interesting to compare the kinetics of action of BCM with those of benzilylcholine and with dibenamine-like drugs. The k_1 of BCM (1.15×10^6 M^{-1} sec^{-1}) is

close to that of benzilylcholine (1.69×10^6 M^{-1} sec^{-1}). The lower value may be due both to the slightly different shape of the quaternary head, and to the dispersal of charge over the ring carbon atoms.

Alkylation of the receptor, once the ethyleniminium ion has combined, appears to be rapid, with a half-time less than 33 sec (probably considerably less). Similar kinetic studies do not appear to have been done with catecholamine antagonists, but there are numerous reports (see 23, 26) that the early phase of dibenamine block is partly reversible and that block in its early stages is reversed by catecholamines which suggest that alkylation does not follow receptor occupation nearly so quickly with these agents as it does with BCM. It is only by applying high concentrations of BCM for very short periods that any reversibility can be observed. Nickerson (27) showed an experiment in which the antihistamine action of compound GD-121 appeared to be partly reversible, and this reversal took about 15 min. If this represents dissociation of reversibly-combined ethyleniminium ion from the receptors it is clear that alkylation must be quite slow, but interpretation is made difficult by the demonstration by Graham and Karrar (24) of high antihistamine activity in the hydrolysis products of some of these compounds. The reversal seen by Nickerson (27) could thus have been due to dissociation of the hydrolysis product, and not of the ethyleniminium ion.

The high rate of alkylation (k_3) with BCM might reflect either a more highly nucleophilic group comprising the anionic site in the muscarinic receptor than in the catecholamine α-receptor, or a closer approach by the ethyleniminium with BCM than with the catecholamine antagonists. The ethyleniminium derivative of BCM is unlikely to be a much more reactive alkylating agent than those of the catecholamine antagonists.

The rate of recovery from BCM block in guinea pig intestinal muscle was about 0.06 hr^{-1}. This is about 1% of the rate at which atropine dissociates from muscarinic receptors in the same tissue (8) and lies within the range of rates of recovery from different alkylating catecholamine antagonists. Dibenamine (one of the most persistent) dissociates at a rate of about 0.03 hr^{-1} in rabbit aortic strips (19) whereas some of the newer compounds (21) appear to be much more rapidly reversible. The reversibility of the block by 2-haloethylamines has been considered by Belleau (25) to indicate that the alkylated group must be a carboxyl or a phosphate group, so that alkylation would lead to the formation of a fairly readily hydrolyzed ester (or, with phosphate, a truly reversible complex). The close similarity between the action of BCM at muscarinic receptors and that of the dibenamine series of antagonists at catecholamine receptors suggests that the same nucleophilic group may form the anionic site of both types of receptor.

The kinetics of action have been interpreted in terms of the rate of reaction of BCM with the receptor, and diffusion barriers such as that introduced in Furchgott's biophase system (28) have not been considered. The finding that occupancy develops exponentially is in itself evidence against penetration of any hypothetical biophase being the rate-determining factor. If we suppose that entry into the biophase (at a rate proportional to the concentration difference) were rate limiting, and that, as in Furchgott's model, reaction with the receptors thereafter was rapid, then occupancy should proceed linearly with time, and not exponentially. Other kinetic studies (8, 9) have led us to reject the biophase hypothesis in intestinal smooth muscle, and the present results provide confirmation. Indeed, studies of the kinetics of onset of irreversible block in catecholamine-stimulated tissues, using solutions of known ethyleniminium concentration (in order to avoid the complications of slow cyclization) should throw more light on the validity of the biophase hypothesis in other tissues.

Our main reason for preparing and studying BCM was to follow up the possibility raised by a recent investigation in this laboratory (8) of labeling muscarinic receptors in intestinal muscle. It was found that

tritiated atropine was taken up by smooth muscle by a mechanism closely related to acetylcholine receptors, but any chemical analysis of the atropine–receptor complex was impossible owing to its instability. The covalently bonded complex between BCM and the receptor should, it is hoped, survive chemical manipulations sufficiently to allow further analysis.

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