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The Conversion of 2-Chloroalkylamine Analogues of Oxotremorine to Aziridinium Ions and Their Interactions with Muscarinic Receptors in the Guinea Pig Ileum

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SUMMARY

Two mustard analogues of oxotremorine, *N*-[4-(2-chloroethylmethylamino)-2-butynyl]-2-pyrrolidone (BM 123) and *N*-[4-(2-chloromethylpyrrolidino)-2-butynyl]-2-pyrrolidone (BM 130), were synthesized. BM 123 and BM 130 cyclize in neutral aqueous solution to aziridinium ions. These aziridinium ions are potent stimulants of the guinea pig ileum. This agonist activity is unaffected by hexamethonium but is inhibited by methylatropine and by pretreatment of solutions of BM 123 and BM 130 with thiosulfate. The parent 2-chloroalkylamines and the alcohols formed by hydrolysis of the aziridinium ions have very weak pharmacological effects. Incubation of the isolated guinea pig ileum for 30 min with BM 123 at 20 μM and 2 μM caused 94% and 48% receptor alkylation, respectively, as calculated from the shift in the agonist dose-response curve. Similar incubations with BM 130 at 20 μM and 5 μM alkylated 85% and 63% of the receptors. No recovery from this apparent blockade was observed over a 4-hr time period. These calculated receptor occupancies by BM 123 and BM 130 agreed with those estimated from reduction of [^{3}H](−)-3-quinuclidinyl benzilate binding to homogenates of the ileum following exposure to BM 123 and BM 130. Methylatropine (20 nM) protected against the irreversible actions of BM 123 and BM 130. In homogenates of the guinea pig ileum, BM 123 and BM 130 also caused a selective reduction in the binding capacity of [^{3}H]*N*-methylscopolamine without significantly affecting the apparent affinity. This inhibitory effect persisted after extensive washing. BM 130 was a weak agonist at nicotinic receptors of the frog rectus abdominis muscle, whereas BM 123 was almost inactive. In conclusion, BM 123 and BM 130 are potent and specific muscarinic agonists that bind irreversibly to muscarinic receptors.

INTRODUCTION

Compounds that bind irreversibly to receptors have been valuable tools for probing drug-receptor interactions. Irreversibly acting compounds may also become useful in attempts to isolate and characterize receptors. For the muscarinic receptor, the development of irreversible ligands has depended almost entirely on structural modifications of antagonists. Thus the nitrogen mustard (*N*-methyl-*N*-2-chloroethylamino) analogue of benzilylcholine (1), its *N*-propyl homologue (2) and some azido derivatives of phenylacetic acid esters (3) and of benzilic acid esters (4) are potent muscarinic antagonists with virtually irreversible actions. The mustard analogue of acetylcholine is a relatively potent agonist at muscarinic receptors in intestinal smooth muscle but shows no (5) or very weak (6) irreversible binding as measured by inhibition of contractile responses. The unexpectedly weak irreversible effects of acetylcholine mustard in whole tissues presumably are due to its enzymatic hydrolysis (7, 8). Robinson *et al.* (8), however, used inhibition of the binding of [^{3}H]propylbenzilylcholine mustard to muscarinic receptors in the guinea pig small intestine, in the presence of a cholinesterase inhibitor, to show that acetylcholine mustard binds virtually irreversibly. Acetylcholine mustard also has pronounced nicotinic effects, since it is equipotent with acetylcholine as a stimulant of the frog rectus abdominis muscle (9).

Oxotremorine is a potent muscarinic agent that is unaffected by cholinesterases and that has very weak nicotinic effects (10, 11). The trimethylammonium ana-

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logue of oxotremorine, oxotremorine-M, and oxotremorine methiodide also possess powerful parasympathomimetic activity (12–14). 2-Chloroalkylamines are known to undergo spontaneous intramolecular cyclization to aziridinium ions with intrinsic alkylating ability (15). We therefore postulated that the mustard analogues of oxotremorine-M (BM 123) and of oxotremorine methiodide (BM 130) (Fig. 1) might be transformed to aziridinium ions (I and II) with ability to alkylate the receptor.

We now present evidence that BM 123 and BM 130 are converted to aziridinium ions and that these are potent muscarinic agonists which bind irreversibly to muscarinic receptors in the isolated guinea pig ileum.

MATERIALS AND METHODS

Synthesis of BM 123, BM 130, and their hydrolysis products. Compound III, *N*-[4-(2-hydroxyethylmethylamino)-2-butynyl]-2-pyrrolidone, was prepared as described by Sterk *et al.* (16) and purified by vacuum distillation; b.p. 169° (0.015 mm Hg). BM 123, *N*-[4-(2-chloroethylmethylamino)-2-butynyl]-2-pyrrolidone hydrochloride, was obtained from Compound III, as previously described (16), in 48% yield. After recrystallization from an ethanol-ether mixture, it had an mp. 140–142°, in agreement with the reported mp. of 142° (16).

Compound IV, *N*-[4-(2-hydroxymethylpyrrolidino)-2-butynyl]-2-pyrrolidone, was prepared through the Mannich reaction from *N*-(2-propynyl)-2-pyrrolidone, 2-hydroxymethylpyrrolidine, paraformaldehyde, and a catalytic amount of cuprous chloride according to the method previously described (17); bp 174° (0.8 mm Hg), yield 54%.

$C_{13}H_{20}N_2O_2$

Calculated: C 66.07, H 8.53, N 11.85
Found: C 65.82, H 8.40, N 11.76

BM 130, *N*-[4-(2-chloromethylpyrrolidino)-2-butynyl]-2-pyrrolidone hydrochloride, was prepared by adding a solution of Compound IV (0.009 mol) in anhydrous chloroform (50 ml) to a solution of thionyl chloride (0.025 mol) in anhydrous chloroform (20 ml). The reaction mixture was stirred at room temperature for 2 hr and was then refluxed for 2 hr. After cooling, the solvent and the excess of thionyl chloride were removed under reduced pressure. The residue, a viscous dark oil, was dissolved in ethanol and boiled with activated charcoal. After filtration on Celite, the solution was concentrated in vacuum, some ether was added, and the crystalline product was recrystallized from an ethanol-ether mixture; m.p. 122–123°, yield 53%.

$C_{13}H_{19}N_2ClO \cdot HCl$

Calculated: C 53.62, H 6.92, Cl 24.35, N 9.62
Found: C 53.35, H 7.20, Cl 24.10 N 9.30

Compound V, *N*-[4-(3-hydroxypiperidino)-2-butynyl]-2-pyrrolidone, was synthesized by the Mannich reaction from *N*-(2-propynyl)-2-pyrrolidone, 3-hydroxypiperidine, and paraformaldehyde as previously described (17); b.p. 179° (0.05 mm Hg), yield 60%.

Measurements of formation and decomposition of aziridinium ions. The method used to quantitate aziridinium ions was based on that of Gill and Rang (1). A 2.0 mM solution of BM 123 or BM 130 in 30 mM sodium-potassium phosphate buffer (pH 7.0) was kept at constant temperature (23° or 37°), and aliquots (5 ml) were removed at various times. The cyclization was stopped by the addition of 0.2 N acetic acid (1 ml). Then 0.5 ml of 0.05 N sodium thiosulfate was added. After 20 min, residual thiosulfate was estimated by titration with standardized potassium triiodide (0.01 N). A starch indicator was used to determine the end-point.

Chloride ion released during the cyclization was measured by argentometric titration according to the method of Volhard (18).

Rate constants were estimated by fitting kinetic models to the data by an unweighted Gauss-Newton nonlinear regression routine programmed on a Hewlett-Packard Model 67 calculator. Chloride release data were fitted to the equation $\lambda = 1 - e^{-k_1 t}$, where λ is the molar proportion of chloride released, k_1 is the apparent first-order rate

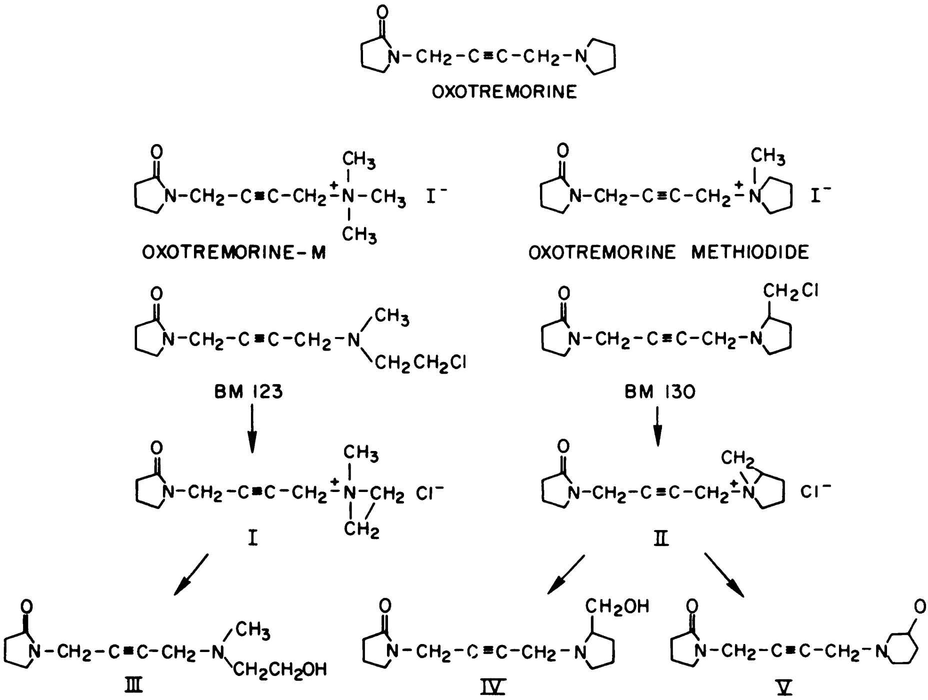

FIG. 1. *Chemical structures of some oxotremorine analogues*

BM 123 and BM 130 are transformed in aqueous solution at neutral pH to the aziridinium ions I and II, respectively. These are then hydrolyzed to the amino alcohols III–V.

constant for the cyclization reaction, and t is time. The concentration of aziridinium ions as a function of time was fitted to the equation

$$\phi = \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t})$$

where ϕ is the molar ratio of aziridinium ion concentration to starting concentration of the parent compound, and k_1 and k_2 are the rate constants for the formation and decay of the aziridinium ion, respectively. Apparent rate constants (k_{app}) for chloride release from BM 130 were estimated over the pH range 5.2–7.6 by fitting the equation $\lambda = 1 - e^{-k_{app}t}$ to data (λ, t) over the time range 0–90 min. The resulting estimates of k_{app} at each pH were then used as input for a regression analysis according to the equation

$$k_{app} = \frac{k_1}{1 + 10^{(pK_a - pH)}}$$

where k_1 is the rate constant for chloride release from the free base of BM 130 and K_a is the acid dissociation constant of BM 130. This analysis provides estimates of pK_a and k_1.

Isolated guinea pig ileum. A standard guinea pig ileum preparation was set up at 37° in Tyrode's solution (137 mM NaCl, 12 mM $NaHCO_3$, 5 mM glucose, 2.7 mM KCl, 1 mM $MgSO_4$, 0.4 mM NaH_2PO_4, and 1.8 mM $CaCl_2$; pH 7.4) as previously described (19).

The muscarinic activity of BM 123 and BM 130 at different times after their dissolution (as 2 mM solutions) in 30 mM sodium-potassium phosphate buffer (pH 7.0) of room temperature was estimated by recording cumulative dose-response curves at intervals over a period of 12 hr. Three different preparations were used for each of these experiments. Potencies at each time point were expressed relative to that of oxotremorine-M, used as a standard agonist. At the time of maximal muscarinic activity, dose-response curves to BM 123 and BM 130 were also obtained in the presence of methylatropine (20 nM) and hexamethonium (0.3 mM).

Separate experiments were performed to examine the effects of prolonged exposure of the ileum to BM 123, BM 130, and oxotremorine-M. For these experiments, BM 123 and BM 130 were cyclized as 2 mM solutions in 50 mM sodium-potassium phosphate buffer (pH 7.4) at room temperature for 60 and 15 min, respectively, when the concentration of aziridinium ions was near maximal. Control responses to oxotremorine-M, oxotremorine, and BM 5 (see below under Drugs) were obtained. The tissue was then exposed to BM 123 (2 μM or 20 μM) or BM 130 (5 μM or 20 μM) for 30 min. All concentrations of BM 123 and BM 130 quoted in this study are those of the parent mustards. After the exposure, the tissue was washed with Tyrode's solution for 60 or 90 min. Sodium thiosulfate (0.1 mM) was always included in the Tyrode's solution during the first 15 min of the wash period in order to destroy any remaining aziridinium ions. Dose-response curves to oxotremorine-M were then obtained at intervals for the next 2.5–3.5 hr. The percentage receptor occupancy (p) by BM 123 or BM 130 was calculated from the ratio of equiactive concentrations of oxotremorine-M at different times after the wash period and before the 30 min exposure (dose ratio), using the relationship 1 (20)

$$p = \frac{(\text{Dose ratio} - 1) \times 100}{\text{Dose ratio}} \qquad (1)$$

Tests for recovery of responses to oxotremorine and BM 5 were performed 2 hr after the exposure to BM 123 or BM 130. Receptor protection experiments were carried out with methylatropine (20 nM), which was allowed to equilibrate with the tissue for 30 min before the addition of BM 123 or BM 130. In control experiments, the ileum was exposed to oxotremorine-M (2 μM and 20 μM) for 30 min. Tests for recovery from desensitization and for subsequent changes in sensitivity of the ileum as a function of time were performed at 15-min intervals for 4.5 hr using oxotremorine-M.

Antagonists (Compounds IV and V) were tested against carbachol and were allowed to equilibrate with the preparation for 10 min. Tests for competitive antagonism were performed according to the procedure of Arunlakshana and Schild (21).

Frog rectus abdominis. A standard frog rectus abdominis (*Rana pipiens*) preparation (22) was set up at 20° in aerated Clark-Ringer solution (110 mM NaCl, 2.4 mM $NaHCO_3$, 10 mM glucose, 1.89 mM KCl, 0.08 mMNaH_2PO_4, and 1.08 mM $CaCl_2$; pH 7.4). The tissue was allowed to rest for 1 hr before being stimulated with carbachol until constant responses were obtained. The preparation was exposed to each drug concentration for 5 min. Responses were expressed as a percentage of the maximal response to carbachol. BM 130 was cyclized for 15 min in a 2 mM solution in 50 mM sodium-potassium phosphate buffer (pH 7.4). BM 123 was cyclized for 1 hr as a 20 mM solution in 100 mM sodium-potassium phosphate buffer (pH 7.4) because of the high concentrations required to record dose-response curves in this preparation.

Muscarinic receptor binding assays. Muscarinic receptor binding assays were run on segments of the guinea pig ileum that had been exposed to BM 123 and BM 130 for 30 min so that a comparison could be made between the estimates of receptor alkylation determined by ^{3}H-ligand binding methods and by application of Eq. 1 as described above. For these experiments, solutions (2 mM) of BM 123 and BM 130 in 50 mM sodium-potassium phosphate buffer (pH 7.4) were incubated at room temperature for 60 and 15 min, respectively, to allow formation of the aziridinium ions. The solutions were put on ice and used immediately. Segments (2.5–3.5 cm) of the guinea pig ileum were preincubated in Tyrode's solution at 37° gassed with 95% O_2 and 5% CO_2 for 15–30 min. An aliquot of BM 123 or BM 130 was added, and the tissue was incubated for an additional 30 min. The incubation was stopped by transferring the ileum to fresh Tyrode's solution containing 1.0 mM thiosulfate. The tissue was washed for 60 min, during which time it was transferred to fresh Tyrode's solution every 20 min. Next, the ileum was mounted on a 1-ml pipette and the outer longitudinal muscle layer was gently rubbed off with a cotton swab. The longitudinal muscle was then minced with scissors and homogenized with a Potter Elvehjem glass homogenizer with teflon pestle in 50 mM sodium-potassium phosphate buffer (pH 7.4) to a concentration representing 10 mg original wet tissue weight/ml phosphate buffer. The tissue was rehomogenized with the Polytron (Brinkmann) at setting 5 for 20 sec and filtered through three layers of cheesecloth. The homogenates were washed twice by centrifugation at 27,000 × g for 10 min followed by resuspension in fresh 50 mM sodium-potassium phosphate buffer (pH 7.4) with the final homogenate concentration being 4.0 mg of original tissue (wet weight) per milliliter of phosphate buffer. This homogenate was assayed subsequently for the binding of [^{3}H](−)-QNB.[3]

In another series of experiments, homogenates of the longitudinal muscle of the guinea pig ileum were incubated with BM 123 and BM 130 to determine what effect this would have on the binding of the specific muscarinic antagonist, [^{3}H]NMS. For these experiments, solutions (2 mM) of BM 123 and BM 130 were incubated at 37° for 40 and 5 min, respectively, to allow formation of the aziridinium ions. The longitudinal muscle was removed from the guinea pig ileum and homogenized in 50 mM phosphate buffer to a concentration of 25 mg of original tissue (wet weight) per milliliter of phosphate buffer as described above. Aliquots (2 ml) of the homogenate were added to small tubes and preincubated at 37° for 10 min in a shaking water bath. Next, BM 123 or BM 130 was added to each tube at a final concentration of 10 μM, and the tubes were incubated for an additional 20 min. The reaction was stopped immediately by addition of atropine (0.1 mM) and thiosulfate (1.0 mM). The homogenates were washed five times by centrifugation at 27,000 × g for 10 min followed by resuspension in 3 ml of phosphate-buffered saline (181 mM Na^+, 100 mM Cl^-, 9.5 mM K^+, 50 mM PO_4, pH 7.4). After the first centrifugation, the homogenates were resuspended in phosphate-buffered saline containing thiosulfate (1.0 mM). After the second, third, and fourth centrifugations, the homogenates were incubated for 5, 10, and 20 min, respectively, to allow time for the dissociation of atropine that might have

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[3] The abbreviations used are: NMS, N-methylscopolamine; QNB, 3-quinuclidinyl benzilate.

been bound tightly to muscarinic receptors. After the fifth centrifugation, the pellets were frozen immediately at −20° and thawed the next day for assay of [^{3}H]NMS binding. The pellets were resuspended with a Potter Elvehjem homogenizer to a concentration representing 1.25 mg of original tissue (wet weight) per milliliter of 50 mM sodium-potassium phosphate buffer.

The specific binding of [^{3}H](−)-QNB was measured according to the rapid filtration method of Yamamura and Snyder (23), and the binding of [^{3}H]NMS was measured by the centrifugation method described by Birdsall *et al.* (24). The following modifications of the assays were made. Aliquots (0.5 ml) of ileal homogenate were incubated with ^{3}H-ligand in a final volume of 2 ml containing 50 mM sodium-potassium phosphate buffer (pH 7.4). The incubation of tissue with [^{3}H](−)-QNB lasted for 60 min at 37°, and that for [^{3}H]NMS lasted for 30 min at 30°. The incubation with [^{3}H]NMS was terminated by centrifugation at 27,000 × *g* for 10 min. Nonspecific binding for both [^{3}H]ligands was defined as the amount of radioactivity measured in the presence of 10 μM atropine. The binding data for [^{3}H]NMS were calculated assuming that both enantiomers of racemic [^{3}H]NMS bound nonspecifically to the same extent but that only the (−)-enantiomer contributed to specific binding. Protein was measured by the method of Lowry *et al.* (25), using bovine serum albumin as the standard.

Drugs. Oxotremorine sesquioxalate (26), oxotremorine-M (12), oxotremorine methiodide (13), and BM 5 [*N*-methyl-*N*-(4-pyrrolidino-1-methyl-2-butynyl)-acetamide oxalate] (27) were prepared as previously described. Other drugs and their sources were the following: carbamylcholine chloride (Aldrich Chemical Company, Milwaukee, Wis.), hexamethonium chloride and methylatropine bromide (K & K Laboratories, Plainview, N. Y.), [^{3}H](−)-QNB (40.2 Ci/mmol), and [^{3}H]NMS (84.8 Ci/mmol) (New England Nuclear Corporation, Boston, Mass.).

RESULTS

Formation and decomposition of aziridinium ions. The cyclization of BM 123 and BM 130 in phosphate buffer (pH 7.0) was monitored by measurement of chloride ion release and by utilizing the quantitative reaction of the formed aziridinium ions with thiosulfate. The latter reaction was also used to follow the hydrolysis of the aziridinium ions. Figure 2 shows the time course for the liberation of chloride ion and for the formation and decay of the aziridinium ion from BM 123 at 37°. The rate constant for the cyclization of BM 123, as measured by chloride release, was 1.14 hr^{-1} at 37° and 0.16 hr^{-1} at 23°. The rate constants for the decay of the aziridinium ion I were 1.98 and 0.56 hr^{-1}, respectively, at 37° and 23°. The aziridinium ion concentration reached its maximum after 40 min at 37° (28%) and after 150 min at 23° (18%).

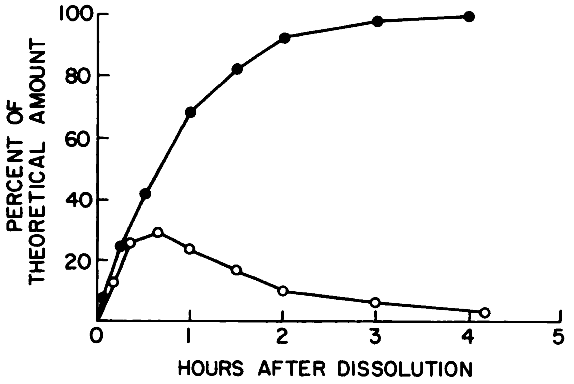

FIG. 2. *Chloride release (●) and aziridinium ion formation and decay (○) from BM 123 at pH 7.0 and 37°*

The ordinate shows concentrations of the ions as percentages of the maximum of 1 Eq/mol of BM 123. Ion concentrations were determined as described under Materials and Methods and are mean values of three separate experiments. The standard errors of the concentrations were always less than 2%.

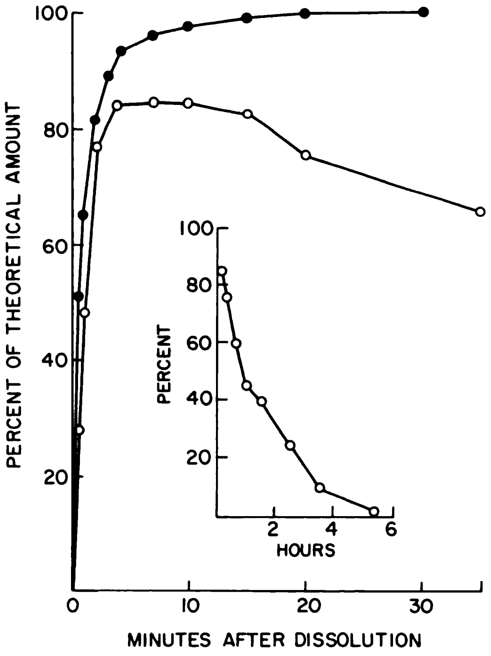

FIG. 3. *Chloride release (●) and aziridinium ion formation and decay (○) from BM 130 at pH 7.0 and 37°*

For details see Fig. 2.

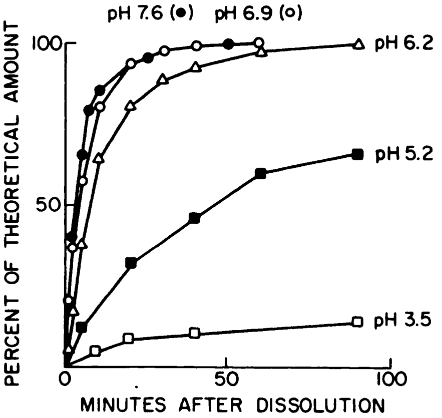

FIG. 4. *pH Dependency of chloride release from BM 130*

Chloride release was measured in phosphate buffer (pH 5.2–7.6) and in water (initial pH 3.5) at 23° as described under Materials and Methods. Data are from single experiments (pH 3.5 and 5.2) or from two separate experiments (pH 6.2, 6.9, and 7.6).

Figure 3 illustrates the time course of chloride ion release and of aziridinium ion formation from BM 130 at 37°. The rate constant for the cyclization of BM 130, as measured from chloride ion release, was 63.6 hr^{-1} at 37° and 10.9 hr^{-1} at 23°. The time course of the decay at 37° of the aziridinium ion derived from BM 130 also is shown in Fig. 3. Rate constants of 0.75 and 0.18 hr^{-1}, respectively, were obtained at 37° and 23°. The aziridinium ion concentration reached its maximum after 5 min at 37° (84%) and after 20 min at 23° (77%).

We also studied the effect of pH on the rate of cyclization of BM 130 at 23° (Fig. 4). The rate of chloride release increased with pH up to pH 7.0 but did not increase much beyond this. The apparent rate constant for chloride release was in excellent agreement with the theoretical equation derived from the assumption that only the free base cyclizes under these conditions. Regression analysis yielded an estimated pK_a value of 6.37 and a first-order rate constant for the free base of 14.2 hr^{-1}.

Identification of the hydrolysis products of BM 130. The hydrolysis of the aziridinium ion II may give rise to two isomeric amino alcohols IV and V (Fig. 1). In order to determine the relative amounts of these, BM 130 (25 mg) was dissolved in 5 ml of 50 mM sodium-potassium phosphate buffer (pH 7.4), and the solution was left at room temperature for 48 hr. The solution was then made faintly alkaline by the addition of 1 N NaOH and extracted with dichloromethane (2 × 10 ml). The organic phase was dried over K_2CO_3. To 1 ml of the dichloromethane solution was added 0.1 ml of bis(trimethylsilyl)trifluoroacetamide reagent (Regis Chemical Company, Morton Grove, Ill.), and the solution was heated in a closed tube at 60° for 1 hr. A volume of 1 μl of this solution was injected into a Hewlett-Packard 5840A gas chromatograph equipped with a flame ionization detector. A silanized glass column (2 m × 2 mm inner diameter) containing 100/120 mesh Supelcoport coated with 5% QF-1 (Supelco Inc., Bellefonte, Pa.) was used. The column temperature was maintained at 200°. Injection port and detector temperatures were 225°. Two sharp peaks were observed (retention times 7.51 and 8.78 min) with relative areas of 5:3. Samples of Compounds IV and V derivatized as above gave single peaks and retention times of 7.50 and 8.75 min, respectively. A sample containing equimolar amounts of Compounds IV and V after derivatization gave two peaks with retention times of 7.50 and 8.75 min and relative areas 1:1. Therefore, both amino alcohols are formed from BM 130, but Compound IV is the more abundant product. In addition to hydrolysis, aziridinium ions in phosphate buffer also are subject to nucleophilic attack by phosphate anion. Thus a phosphate adduct has been shown to be a decomposition product of propylbenzilylcholine mustard in phosphate buffer (28). The extent of formation of such an adduct from BM 123 and BM 130 is not known.

Isolated guinea pig ileum. BM 123 and BM 130 were potent stimulants of the guinea pig ileum. The contractile responses elicited by the two coupounds resembled those of oxotremorine-M and oxotremorine methiodide (Fig. 5). The muscarinic activity of solutions of BM 123 and BM 130 in phosphate buffer (pH 7.0) varied greatly with time after dissolution. For BM 123, maximal muscarinic activity was observed with a solution kept at room temperature for 2–4 hr, whereas solutions of BM 130 showed maximal activity 15–60 min after dissolution (Fig. 6). The short exposure (about 1.5 min) of the ileum to the low concentrations of BM 123 and BM 130 required to record dose-response curves did not alter the muscle responses to oxotremorine-M; i.e., no alkylation of the receptors was detected under these conditions. Therefore, BM 123 and BM 130 were bioassayed against oxotremorine-M. Relative potencies of BM 123 and BM 130 were obtained at the time of peak activity. The results are summarized in Table 1 and Fig. 5, which also include oxotremorine methiodide and carbachol. Methylatropine (20 nM) increased the ED_{50} values of BM 123 and BM 130 28- to 30-fold, whereas hexamethonium (0.3 mM) was without effect.

The muscarinic activity of solutions of BM 123 and BM 130 in phosphate buffer (pH 7.0) over a time period of 12-hr closely paralleled the aziridinium ion concentration as determined by thiosulfate consumption (Fig. 6). Forty-eight hours after dissolution, no aziridinium ion was detected in solutions of BM 123 and BM 130. At this time, solutions of BM 123 still showed weak muscarinic activity (less than 1% of the maximal activity), whereas solutions of BM 130 did not exhibit such activity. Treatment of a solution of BM 123 that had been kept at room temperature and pH 7.0 for 3 hr, and which therefore had maximal muscarinic activity (Fig. 6), with sodium thiosulfate (0.1 mM for 15 min) almost completely abolished the muscarinic response. Since this solution had liberated only 40% of the theoretical amount of chloride (data not shown), it still contained about 60% of the original concentration of the parent mustard. Thiosulfate treatment of a solution of BM 130, which had been kept at room temperature for 30 min, totally abolished the muscarinic response. This solution had liberated virtually all the chloride (Fig. 4) and therefore did not contain the parent mustard. On the other hand, sodium thiosulfate (10 μM) added to the organ bath did not protect against the muscarinc actions of BM 123 and BM 130. Finally, thiosulfate treatment had no effect on the muscarinic activity of a solution of oxotremorine-M.

The alcohol (III) formed by hydrolysis of BM 123 was a muscarinic agonist that had less than 0.1% of the potency of oxotremorine-M. Both alcohols formed by hydrolysis of BM 130 were weak antagonists to carbachol on the isolated guinea pig ileum. Compound IV behaved like a competitive antagonist with a dissociation constant (K_b) of 11.9 ± 0.8 μM (SE, $n = 4$). Compound V appeared to be a noncompetitive antagonist with a K_B value of 295 ± 60 μM (SE, $n = 4$).

To investigate possible alkylation of muscarinic receptors, segments of the isolated guinea pig ileum were exposed to BM 123 or BM 130 for 30 min. The length of the muscle was monitored continuously during this exposure. After the initial contraction, the length of the muscle increased steadily and in some experiments returned to near resting length before the end of the 30 min exposure period. In other experiments, the resting length was not restored during the 30-min exposure but was restored during the subsequent wash period. In some experiments, in which the muscle was exposed to the higher concentration (20 μM) of BM 123 and BM 130, there was a decrease in the resting length which was not influenced by washing or by atropine (1 μM). A similar phenomenon has been previously noted with acetylcholine mustard (8). The latter experiments were discarded.

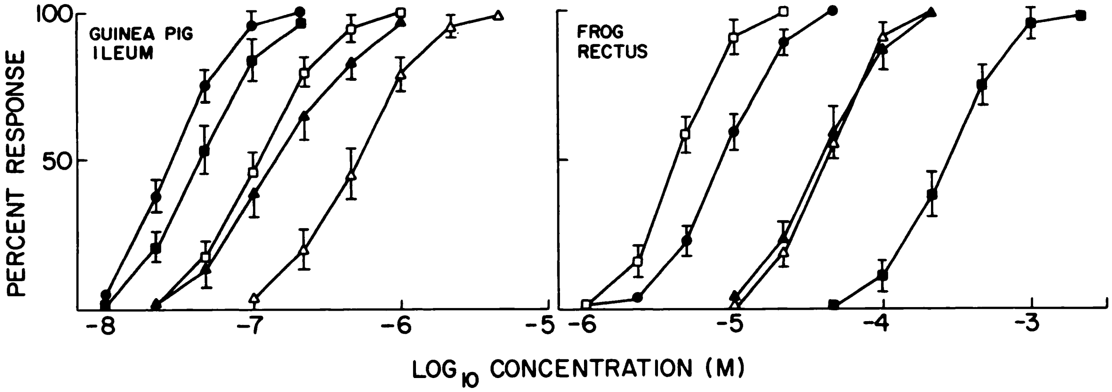

Fig. 5. *Dose-response curves in the isolated guinea pig ileum and in the frog rectus abdominis muscle of oxotremorine-M (●), BM 123 (■), carbachol (□), BM 130 (▲), and oxotremorine methiodide (△)*

Responses are expressed as a percentage of the maximum contraction elicited by oxotremorine-M (guinea pig ileum) or carbachol (frog rectus). Vertical bars show standard error. The number of preparations used is given in Table 1.

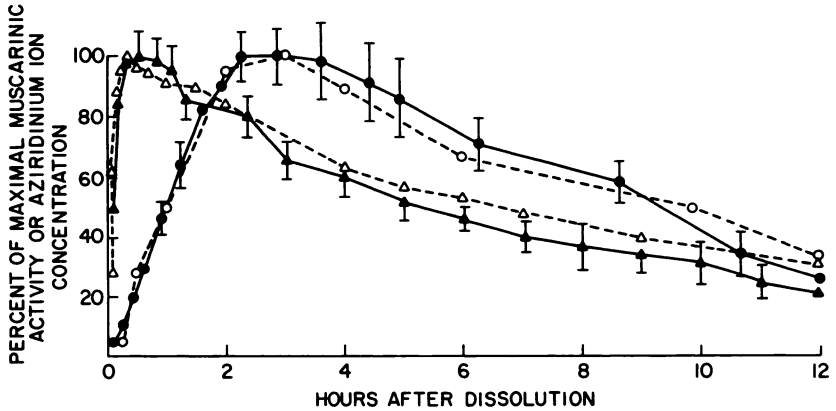

Fig. 6. *Correlation of muscarinic activity and aziridinium ion concentration in solutions of BM 123 and BM 130*

The solutions were kept in phosphate buffer at pH 7.0 and 23°. The muscarinic activity (●) and the aziridinium ion concentration (○) in the solution of BM 123 are expressed in terms of the muscarinic activity and the aziridinium ion concentration of a sample taken at 3 hr. For the solution of BM 130, the muscarinic activity (▲) and the aziridinium ion concentration (△) are given relative to those of a sample withdrawn at 20 min. Vertical bars show standard error of three or four experiments.

Table 1

Muscarinic and nicotinic activity of some oxotremorine analogues

The muscarinic and nicotinic activities are expressed as equipotent molar ratios (EPMR) ± standard error relative to oxotremorine-M. The number of preparations used is given in parentheses.

Compound	Isolated guinea pig ileum EPMR	Frog rectus abdominis EPMR	ED_{50} Frog rectus/ ED_{50} guinea pig ileum
BM 123	1.4 ± 0.12 (6)	30.2 ± 2.4 (4)	6590
BM 130	5.6 ± 0.58 (6)	4.9 ± 0.79 (4)	270
Oxotremorine Methiodide	19.1 ± 2.0 (4)	5.0 ± 0.42 (4)	81
Carbachol	3.9 ± 0.30 (5)	0.48 ± 0.04 (4)	36
Oxotremorine-M	1.0	1.0	288

Recovery from desensitization caused by exposure of the ileum to oxotremorine-M at 2 μM and 20 μM was complete within 50 and 80 min, respectively. Therefore, tests for recovery of responses to oxotremorine-M after exposure to BM 123 at 2 μM and BM 130 at 5 μM were begun after a wash period of 60 min. After exposure to a concentration of 20 μM of BM 123 and BM 130, the wash period was extended to 90 min. From the dose-response curves to oxotremorine-M before and at different times after the exposure of the ileum to BM 123 and BM 130, the percentage receptor occupation by BM 123 and BM 130 was calculated (Table 2). Corrections were made for the small changes in the sensitivity of the ileum to oxotremorine-M occurring with time as evidenced in the control experiments. BM 123 and BM 130 caused a dose-dependent decrease of the sensitivity of the guinea pig ileum to oxotremorine-M. No significant recovery of responses to oxotremorine-M was observed over a time period of 4.5 hr after the exposure.

TABLE 2

Percentage occupancy of muscarinic receptors in the isolated guinea pig ileum by BM 123 and BM 130

The ileum was exposed to each concentration of BM 123 and BM 130 for 30 min. In the protection experiments, methylatropine (20 nM) was allowed to equilibrate with the preparation for 30 min before the addition of BM 123 or BM 130. Each value represents the mean ± standard error. The number of animals used is given in parentheses.

Tissue treatment	% Receptor occupancy						% Decrease of response to BM 5[b]
	Inhibition of contractile responses after exposure of					Inhibition of [^{3}H]QNB binding[a]	
	1.0 hr	1.5 hr	2.5 hr	3.5 hr	4.5 hr		
BM 123, 20 μM (4)		94.0 ± 3.1	94.0 ± 2.9	92.8 ± 2.8	92.3 ± 2.1	81.9 ± 1.8	100
BM 123, 2 μM (5)	49.4 ± 6.9	48.0 ± 8.0	49.4 ± 6.4	50.6 ± 4.9		61.0 ± 2.7	63 ± 8
Methylatropine and BM 123, 20 μM (3)		32.3 ± 5.8	36.4 ± 2.6	39.6 ± 2.1	39.3 ± 1.8		61 ± 5
Methylatropine and BM 123, 2 μM (3)	11.7 ± 2.7	8.9 ± 4.7	4.9 ± 4.9				17 ± 6
BM 130, 20 μM (4)		85.4 ± 2.8	85.6 ± 2.9	85.8 ± 2.6	86.2 ± 2.7	79.8 ± 3.0	100
BM 130, 5 μM (3)	64.5 ± 2.0	62.9 ± 3.5	61.3 ± 6.4	62.1 ± 5.9		66.5 ± 2.5	69 ± 7
Methylatropine and BM 130, 20 μM (3)		38.6 ± 10.9	38.3 ± 8.5	39.4 ± 6.7	41.8 ± 6.5		66 ± 6
Methylatropine and BM 130, 5 μM (3)	27.9 ± 1.8	25.3 ± 5.5	23.9 ± 3.5				26 ± 3

[a] Percentage inhibition of specific [^{3}H](−)-QNB binding as compared to untreated tissue.

[b] Percentage decrease of the height of the contractile response as compared to the height of the response before incubation with BM 123 or BM 130.

A test of the specificity of action of BM 123 and BM 130 was made by measuring the protecting action of methylatropine against the irreversible effects. Methylatropine (20 nM) significantly reduced the fraction of receptors occupied by BM 123 at 20 μM and by BM 130 at 20 μM and 5 μM and almost fully protected against the irreversible actions of BM 123 at 2 μM (Table 2).

We also studied changes in the height of the contractile responses of oxotremorine-M, oxotremorine, and BM 5, a partial muscarinic agonist (27), caused by the treatments of the ileum with BM 123 and BM 130 described above. In this way, we hoped to obtain an independent verification of the method used to determine the extent of receptor alkylation. The maximal response to oxotremorine-M was not depressed in any of the experiments summarized in Table 2. The response to oxotremorine was always depressed after exposure of the ileum to BM 123 at 20 μM and in two of the four experiments in which the ileum was exposed to BM 130 at 20 μM. In all other experiments, the maximal response to oxotremorine was virtually unaffected. Finally, the response to BM 5 was abolished in two experiments and depressed in all others, although the depression was small in those experiments in which methylatropine protected against extensive receptor alkylation (Table 2).

Frog rectus abdominis. BM 123 and BM 130 as well as oxotremorine-M and oxotremorine methiodide caused slow contractions of the frog rectus abdominis muscle similar to those elicited by carbachol (Fig. 5). The equipotent molar ratios relative to oxotremorine-M are summarized in Table 1, which also shows the ratio of the ED_{50} values for contraction of the frog rectus and of the guinea pig ileum for each compound. This ratio was calculated to obtain a measure of the selectivity for muscarinic receptors. There was no clear evidence of irreversible actions of BM 123 or BM 130 under the conditions used to record dose-response curves in the frog rectus abdominis preparation.

Muscarinic receptor binding activity. Treatment of the guinea pig ileum with BM 123 and BM 130 caused an irreversible inhibition of the binding of [^{3}H](−)-NMS. Homogenates of the longitudinal muscle of the guinea pig ileum were incubated with BM 123 and BM 130 (10 μM) for 20 min and then washed extensively (see Materials and Methods). Control homogenates were treated identically except for exposure to BM 123 and BM 130. Measurements of the specific binding of [^{3}H](−)-NMS to the homogenates were made at racemic [^{3}H]ligand concentrations of 0.069, 0.17, 0.44, 1.1, and 2.8 nM, and the results were analyzed by nonlinear regression analysis to determine the effect of incubation of BM 123 and BM 130 on the dissociation constant and binding capacity of [^{3}H](−)-NMS. The control binding isotherm was consistent with a Langmuir isotherm having a dissociation constant of 0.18 nM and binding capacity of 1.52 pmol/mg of protein. Prior treatment of the homogenates with BM 123 and BM 130 caused a significant reduction in the binding capacity to 0.656 and 0.851 pmol/mg of protein, respectively, without significantly affecting the apparent affinity of [^{3}H](−)-NMS. Nonlinear regression and analysis of variance showed no significant increase in residual error ($F = 0.905$, $p = 0.438$) when the data were fitted sharing the control estimate of the dissociation constant among the three binding isotherms. Figure 7 summarizes these data in the form of a Scatchard plot. We have found a greater reduction in receptor capacity after an incubation of tissue with BM 123 or BM 130 when the reaction was not stopped immediately by addition of atropine.

Binding assays with [^{3}H](−)-QNB were run on ilea which had been incubated with BM 123 or BM 130 for 30 min to determine whether the extent of receptor

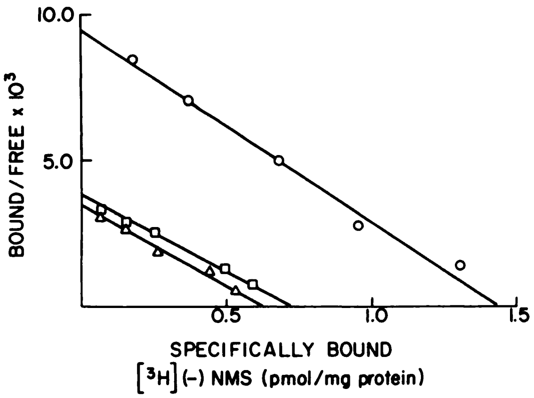

FIG. 7. *Scatchard analysis of the specific binding of [^{3}H](−)-NMS to the longitudinal muscle of the guinea pig ileum from control (○), BM 123-treated (△), and BM 130-treated (□) tissue*

The tissues were treated with BM 123 or BM 130 at 10 μM for 20 min as described under Materials and Methods. The data were calculated from the mean binding values of three experiments, each performed in triplicate.

alkylation determined pharmacologically was comparable to that estimated by measuring the reduction in [^{3}H](−)-QNB binding. Segments of the guinea pig ileum were incubated with BM 123 (2 μM and 20 μM) and BM 130 (5 μM and 20 μM) for 30 min and then washed extensively as described under Materials and Methods. Control ilea were treated the same way except for exposure to BM 123 and BM 130. [^{3}H](−)-QNB binding was measured at a concentration of 0.4 nM in homogenates of the longitudinal muscle of the guinea pig ileum. The percentage of receptors alkylated by BM 123 and BM 130 was calculated as the percentage inhibition of specific [^{3}H](−)-QNB binding. Since experiments with [^{3}H]NMS showed that the inhibitory effect of BM 123 and BM 130 on muscarinic receptor binding properties was due to a selective reduction in binding capacity without an affect on affinity, it was assumed that the reduction in the binding of [^{3}H](−)-QNB was proportional to the decline in receptor capacity. The results of these experiments are shown in Table 2. It can be seen that there is general agreement among the various estimates of receptor alkylation caused by treatment of the ileum with BM 123 and BM 130.

DISCUSSION

BM 123 and BM 130 cyclize in aqueous solution at neutral pH to form aziridinium ions, concurrently liberating one equivalent of chloride ion. The kinetics of the formation of the aziridinium ion differed markedly for the two compounds. Chloride ion was released extremely slowly from BM 123. The rate constant for the cyclization of BM 123, as measured by chloride release, was lower than the rate constant for the decay of the aziridinium ion I. Furthermore, at 37° only about 50% of the theoretical amount of chloride ion had been released from BM 123 at the time of maximum aziridinium ion concentration (Fig. 2). These observations satisfactorily explain the relatively low conversion of BM 123 to I and suggest no need to invoke competing reactions, e.g., reaction of BM 123 with the aziridinium ion to form a piperazinium dimer (15). In contrast, chloride ion was released rapidly from BM 130, leading to a relatively higher peak concentration of the aziridinium ion II (Fig. 3).

A number of observations suggest that the aziridinium ions I and II are the molecular species mainly responsible for the muscarinic actions of BM 123 and BM 130. For example, the *in vitro* muscarinic activity of solutions of BM 123 and BM 130 at different stages in the cycle of generation and hydrolysis of the aziridinium ions closely paralleled the aziridinium ion concentrations as determined by thiosulfate consumption (Fig. 6). Thiosulfate treatment of a solution of BM 123, which showed maximal muscarinic activity, almost completely abolished the activity. Since this solution contained about 60% of the original amount of BM 123 (See Results), and since thiosulfate does not react directly with 2-chloroethylamines, the disappearance of the muscarinic activity after treatment with thiosulfate not only points to the aziridinium ion as the active species, but also suggests that BM 123 has very weak, if any, muscarinic activity. Similar treatment of a solution of BM 130 completely abolished the muscarinic activity. However, this observation does not rule out possible muscarinic activity of BM 130 (see Results). Since thiosulfate added to the organ bath did not protect against the muscarinic actions of BM 130, it appears that the interaction of the aziridinium ion II with muscarinic receptors proceeds more rapidly than its reaction with thiosulfate. Considering the rapid cyclization of BM 130, it is therefore difficult to prove unequivocally the inactivity of the parent 2-chloroalkylamine. However, from structure-activity relationships in other analogues of oxotremorine, including the 2-methylpyrrolidino (29) and the 2-hydroxymethylpyrrolidino (this work) derivatives, one would expect BM 130 to have antagonistic rather than agonistic properties. Moreover, BM 130 (pK_a 6.37) is largely unprotonated at physiological pH, and it is known that the muscarinic activity of tertiary amines is associated with their protonated forms (13). Finally, the alcohol formed by hydrolysis of BM 123 (i.e., III) is a very weak muscarinic agent, whereas the alcohols derived from BM 130 (i.e., IV and V) are weak muscarinic antagonists.

BM 123 was only slightly less potent than oxotremorine-M (Table 1) and equipotent with oxotremorine in the guinea pig ileum. If one takes into account the poor yield of the aziridinium ion I from BM 123, then I should be more potent than oxotremorine-M. BM 130 was 3–4 times more potent than oxotremorine methiodide. It therefore appears that the minor structural modification of connecting two methyl groups of oxotremorine-M or of connecting the methyl group with the α-carbon of the pyrrolidine ring of oxotremorine methiodide increases muscarinic activity. At nicotinic receptors of the frog rectus abdominis muscle, this structural modification reduces the activity of oxotremorine-M but has no effect on the activity of oxotremorine methodide (Table 1). The selectivity of these compounds for muscarinic as opposed to nicotinic receptors may be expressed as the ratio of the ED_{50} values for contraction of the frog rectus and of the guinea pig ileum (Table 1). BM 123 has the highest selectivity, being about 175-fold more selective for muscarinic receptors than carbachol. The low nicotinic activity of BM 123 is in sharp contrast to the profound nicotinic effects of acetylcholine mustard (9, 30).

In homogenates of the guinea pig ileum, BM 123 and BM 130 caused a selective reduction in the binding capacity of [^{3}H]NMS without significantly affecting the apparent affinity (Fig. 7). This inhibitory effect persisted after extensive washing, indicating a covalent interaction of the aziridinium ions of BM 123 and BM 130 with ileal muscarinic receptors. These results and the high potency of the aziridinium ions in stimulating contractions of the ileum suggested that, with higher concentrations and/or prolonged exposure, alkylation of receptors could occur in whole ileum, leading to a persistent functional blockade. An often-used measure of drug antagonism is the agonist dose ratio, i.e., the ratio by which the agonist concentration must be increased in order to produce a standard response after the addition of the antagonist. The fraction of receptors occupied by the antagonist may be calculated from the agonist dose ratio using the relationship 1. However, there are experimental difficulties involved in using agonist dose ratios calculated from contractile responses as a measure of irreversible binding of a stimulant drug. Prolonged exposure to high bath concentrations of such a drug will cause receptor desensitization that initially may be indistinguishable from irreversible binding. Also, the relationship 1 is valid only if the agonist used to measure dose ratios occupies a negligible fraction of the total amount of receptors available. In the present study, the time required for recovery from desensitization caused by oxotremorine-M was used as an approximate measure of the recovery from desensitization after exposure to BM 123 and BM 130. Oxotremorine-M was also the agonist used to measure dose ratios, since it has a very large spare receptor capacity in the guinea pig ileum (31). With this method, BM 123 and BM 130 (20 μM for 30 min) were found to occupy permanently 94% and 85%, respectively, of the receptors. Strong support for the validity of these calculated receptor occupancies comes from the observation that the maximal response to oxotremorine, which must occupy 10–15% of the receptors for maximal response (29), was slightly depressed. Furthermore, the maximal response to the partial agonist, BM 5, which must occupy virtually all of the receptors for its maximum response (32), was completely abolished (Table 2). The lower receptor occupancies after exposure to 2 μM BM 123 and to 5 μM BM 130 and after the protection experiments were confirmed by the observations that the maximal response to oxotremorine was unaffected and that the response to BM 5 was depressed, the depression roughly paralleling the extent of receptor alkylation. Finally, the calculated receptor occupancies following exposure of the ileum to BM 123 and BM 130 showed general agreement with those estimated by reduction of [^{3}H](−)-QNB binding to homogenates (Table 2). The latter observation contrasts with some recent results obtained in the guinea pig ileum with the irreversible antagonist benzilylcholine mustard. Major discrepancies were found between muscarinic receptor occupancy by benzilylcholine mustard determined from shifts in the dose-response curve to an agonist and from inhibition of [^{3}H]QNB binding (33).

We conclude on the basis of the above results that BM 123 and BM 130 cause a dose-dependent, persistent decrease in sensitivity of the guinea pig ileum to muscarinic agonists, presumably through covalent interaction with the muscarinic receptor. This functional blockade appears to be specific, since it is prevented by low concentrations of methylatropine and since it occurs at concentrations that have no nicotinic effects on frog rectus. The mechanism of this apparent blockade has yet to be established. For example, it is not known whether covalent binding of BM 123 and BM 130 results in an active or inactive receptor complex. As previously pointed out (34), it is possible that the receptor remains in an activated state after alkylation by the aziridinium ions. A persistent desensitization could then result in a functional blockade similar to that caused by an irreversibly bound antagonist. In contrast, the irreversible binding of bromoacetylcholine (35) and chloroxymorphamine (36) to nicotinic and opioid receptors, respectively, leads to a sustained tissue response. These agents, however, are believed to bind covalently at a noncritical site near the receptor (35, 36). Presumably, BM 123 and BM 130 alkylate a nucleophilic group at the agonist recognition site proper.

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REFERENCES

1. Gill, E. W., and H. P. Rang. An alkylating derivative of benzilylcholine with specific and long-lasting parasympatholytic activity. *Mol. Pharmacol.* **2:**284–297 (1966).
2. Young, J. M., R. Hiley, and A. S. V. Burgen. Homologues of benzilylcholine mustard. *J. Pharm. Pharmacol.* **24:**950–954 (1972).
3. Moreno-Yanes, J. A., and H. R. Mahler. Photoaffinity labeling of specific muscarinic antagonist binding sites of brain. I. Preliminary studies using two *p*-azidophenylacetate esters of tropine. *Biochem. Biophys. Res. Commun.* **92:**610–617 (1980).
4. Amitai, G., S. Avissar, D. Balderman, and M. Sokolovsky. Affinity labeling of muscarinic receptors in rat cerebral cortex with photolabile antagonist. *Proc. Natl. Acad. Sci. U. S. A.* **79:**243–247 (1982).
5. Hirst, M., and C. H. Jackson. The conversion of methyl-2-acetoxyethyl-2′-chloroethylamine to an acetylcholine-like aziridinium ion and its action on the isolated guinea pig ileum. *Can. J. Physiol. Pharmacol.* **50:**798–808 (1972).
6. Hudgins, P. M., and J. F. Stubbins. A comparison of the action of acetylcholine and acetylcholine mustard (chloroethylmethylaminoethyl acetate) on muscarinic and nicotinic receptors. *J. Pharmacol. Exp. Ther.* **182:**303–311 (1972).
7. Hudgins, P. M., and J. F. Stubbins. Interactions of acetylcholine mustard with acetylcholinesterase. *J. Pharm. Sci.* **64:**1419–1421 (1975).
8. Robinson, D. A., J. G. Taylor, and J. M. Young. The irreversible binding of acetylcholine mustard to muscarinic receptors in intestinal smooth muscle of the guinea pig. *Br. J. Pharmacol.* **53:**363–370 (1975).
9. Willcockson, W. S., M. Kahlid, A. E. Ahmed, and G. R. Hillman. Effects of acetylcholine mustard analogs on schistosome and vertebrate neuromuscular preparations. *J. Pharmacol. Exp. Ther.* **218:**330–336 (1981).
10. Cho, A. K., W. L. Haslett, and D. J. Jenden. The peripheral actions of oxotremorine, a metabolite of tremorine. *J. Pharmcol. Exp. Ther.* **138:**249–257 (1962).
11. Ringdahl, B. A comparison of the stimulant activities of oxotremorine analogues on the frog rectus abdominis and the guinea pig ileum. *Eur. J. Pharmacol.* **99:**177–184 (1984).
12. Bebbington, A., R. W. Brimblecombe, and D. Shakeshaft. The central and peripheral activity of acetylenic amines related to oxotremorine. *Br. J. Pharmacol.* **26:**56–67 (1966).
13. Hanin, I., D. J. Jenden, and A. K. Cho. The influence of pH on the muscarinic action of oxotremorine, arecoline, pilocarpine and their quaternary ammonium analogs. *Mol. Pharmacol.* **2:**352–359 (1966).
14. Resul, B., B. Ringdahl, R. Dahlbom, and D. J. Jenden. Muscarinic activity of some secondary and tertiary amines and quaternary ammonium salts structurally related to oxotremorine. *Eur. J. Pharmacol.* **87:**387–396 (1983).

15. Graham, J. D. P. 2-Halogenoalkylamines. *Prog. Med. Chem.* **2**:132–175 (1962).
16. Sterk, L., G. Deak, and L. Gyorgy. Synthesis of 1,4-diaminobutyne derivatives containing the 2-chloroethylamino group. *Acta Chim. Acad. Sci. Hung.* **77**:109–113 (1973).
17. Karlen, B., B. Lindeke, S. Lindgren, K.-G. Svensson, R. Dahlbom, D. J. Jenden, and J. E. Giering. Acetylene compounds of potential pharmacological value. XIV. *N*-(*t*-Aminoalkynyl)-substituted succinimides and maleimides: a class of central anticholinergic agents. *J. Med. Chem.* **13**:651–657 (1970).
18. Kolthoff, I. M., and V. A. Stenger. *Volumetric Analysis*, Vol. 2. Wiley Interscience, New York, 239–300 (1947).
19. Ringdahl, B., B. Resul, D. J. Jenden, and R. Dahlbom. Muscarinic activity in the isolated guinea pig ileum of some carboxamides related to oxotremorine. *Eur. J. Pharmacol.* **85**:79–83 (1982).
20. Paton, W. D. M. A theory of drug action based on the rate of drug receptor combination. *Proc. R. Soc. Lond. B Biol. Sci.* **154**:21–69 (1961).
21. Arunlakshana, O., and H. O. Schild. Some quantitative uses of drug antagonists. *Br. J. Pharmacol.* **14**:48–58 (1959).
22. Edinburgh Pharmacology Department Staff. *Pharmacological Experiments on Isolated Preparations.* Livingstone, Edinburgh, 38–40 (1968).
23. Yamamura, H. I., and S. H. Snyder. Muscarinic cholinergic binding in rat brain. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1725–1729 (1974).
24. Bridsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.* **14**:723–736 (1978).
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
26. Bebbington, A., and D. Shakeshaft. An improved synthesis of oxotremorine. *J. Med. Chem.* **8**:274–275 (1965).
27. Resul, B., R. Dahlbom, B. Ringdahl, and D. J. Jenden. *N*-Alkyl-*N*-(4-*tert*-amino-1-methyl-2-butynyl)carboxamides, a new class of potent oxotremorine antagonists. *Eur. J. Med. Chem.* **17**:317–322 (1982).
28. Young, J. M. Pathways of decomposition of propylbenzilylcholine mustard in neutral and alkaline solution. *J. Pharm. Pharmacol.* **34**:162–167 (1982).
29. Ringdahl, B., and D. J. Jenden. Affinity, efficacy, and stereoselectivity of oxotremorine analogues for muscarinic receptors in the isolated guinea pig ileum. *Mol. Pharmacol.* **23**:17–25 (1983).
30. Clement, J. G., M. Hirst, and E. H. Colhoun. Comparative toxicity of acetylcholine mustard (methyl-2-acetoxyethyl-2′-chloroethylamine) in the mouse and American cockroach. *J. Agric. Food Chem.* **22**:873–876 (1974).
31. Ringdahl, B. Affinity and efficacy of oxotremorine analogues at ileal muscarinic receptors, in *Dynamics of Cholinergic Function* (I. Hanin, ed.). Plenum Press, New York (in press).
32. Ringdahl, B. Determination of dissociation constants and relative efficacies of oxotremorine analogues at muscarinic receptors in the guinea pig ileum by pharmacological procedures. *J. Pharmacol. Exp. Ther.* **229**:199–206 (1984).
33. Siegel, H., and D. J. Triggle. Benzilylcholine mustard and spare receptors in guinea pig ileum. *Life Sci.* **30**:1645–1652 (1982).
34. Ehlert, F. J., D. J. Jenden, and B. Ringdahl. An alkylating derivative of oxotremorine interacts irreversibly with the muscarinic receptor. *Life Sci.* **34**:985–991 (1984).
35. Silman, I., and A. Karlin. Acetylcholine receptor: covalent attachment of depolarizing groups at the active site. *Science* (*Wash. D. C.*) **164**:1420–1421 (1969).
36. Caruso, T. P., D. L. Larson, P. S. Portoghese, and A. E. Takemori. Pharmacological studies with an alkylating narcotic agonist, chloroxymorphamine, and antagonist, chlornaltrexamine. *J. Pharmacol. Exp. Ther.* **213**:539–544 (1980).

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