

# Conversion of *N*-(2-Chloroethyl)-4-piperidinyll Diphenylacetate (4-DAMP Mustard) to an Aziridinium Ion and Its Interaction with Muscarinic Receptors in Various Tissues

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## SUMMARY

A 2-chloroethylamine derivative [*N*-(2-chloroethyl)-4-piperidinyll diphenylacetate (4-DAMP mustard)] of the selective muscarinic antagonist *N,N*-dimethyl-4-piperidinyll diphenylacetate (4-DAMP) was synthesized, and its conversion to an aziridinium ion and interaction with muscarinic receptors was investigated. When dissolved in aqueous solution at pH 7.4 and 37°, 4-DAMP mustard released an equivalent amount of chloride. The release of chloride was consistent with a first-order process having a half-time of 5.7 min. The aziridinium ion reached a peak concentration at 32 min, corresponding to 75% of the initial concentration of 4-DAMP mustard. When homogenates of rat brain, heart, and submaxillary gland were incubated with 4-DAMP mustard (9 nM) for 1 hr, washed extensively, and then assayed for muscarinic receptor binding properties, a 56% decrease in the binding capacity of *N*-[<sup>3</sup>H]methylscopolamine in the heart and brain and a 71% decrease in the gland were observed, without a significant change in the dissociation constants. The affinity of 4-DAMP mustard and its transformation products for muscarinic receptors

was determined in competitive binding experiments with *N*-[<sup>3</sup>H]methylscopolamine, and the results show that the aziridinium ion of 4-DAMP mustard was the most potent form, compared with the parent 2-chloroethylamine (4-DAMP mustard) and the alcoholic hydrolysis product. The rates of receptor alkylation by 4-DAMP mustard were measured in the rat heart and gland. Virtually no alkylation (<1%) occurred in the heart at a 4-DAMP mustard concentration of 1.6 nM, after 30 min, whereas almost 50% alkylation was observed in the gland under the same conditions. Almost complete alkylation of receptors in the gland could be achieved at a 4-DAMP mustard concentration of 200 nM, after 1 hr. Treatment of the isolated rat ileum with 4-DAMP mustard caused an irreversible blockade of contractions elicited by the muscarinic agonist oxotremorine-M, and this blockade persisted after extensive washing. The results presented here show that 4-DAMP mustard forms an aziridinium ion that binds irreversibly to muscarinic receptors and exhibits selectivity for M<sub>3</sub>, compared with M<sub>2</sub> muscarinic receptors.

Nitrogen mustard compounds (2-halo-ethylamines) spontaneously form reactive aziridinium ion derivatives at neutral pH. This reactive moiety has been the functional basis of many irreversible alkylating agents. Previously, several irreversible muscarinic agonists and antagonists, including benzilycholine mustard (1) and its homologues (2), acetylcholine mustard (3), and the nitrogen mustard analogues of oxotremorine, BM 123 and BM 130 (4), have been made by substituting a 2-haloalkyl group on the basic nitrogen atom of the compound, and these agents have been useful in investigating drug-receptor interactions.

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To date, the muscarinic class of receptors has been divided into three subtypes, based on their pharmacological properties (M<sub>1</sub>-M<sub>3</sub>); however, five genes for the receptor have been cloned (m<sub>1</sub>-m<sub>5</sub>) (5). Based on the binding properties of the expressed recombinant muscarinic receptors and the distribution of their mRNA, it appears that the molecularly defined receptor subtypes m<sub>1</sub>, m<sub>2</sub>, and m<sub>3</sub> represent the pharmacologically defined M<sub>1</sub>, M<sub>2</sub>, and M<sub>3</sub> subtypes, respectively (6-9). Because this heterogeneity of the muscarinic receptors could potentially complicate studies, selective irreversible alkylating compounds would be valuable in characterizing receptors in certain tissues containing a mixed population of subtypes.

Recently, a nitrogen mustard analogue of the reversible muscarinic antagonist 4-DAMP was synthesized (see Fig. 1) and reported to exhibit pharmacological selectivity similar to that of 4-DAMP in isolated tissues (10). 4-DAMP has been shown

**ABBREVIATIONS:** 4-DAMP, *N,N*-dimethyl-4-piperidinyll diphenylacetate; 4-DAMP mustard, *N*-(2-chloroethyl)-4-piperidinyll diphenylacetate; TLC, thin layer chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetracetic acid; NMS, *N*-methylscopolamine; KRB, Kreb's Ringer bicarbonate; Boc, *N*-*tert*-butoxycarbonyl.

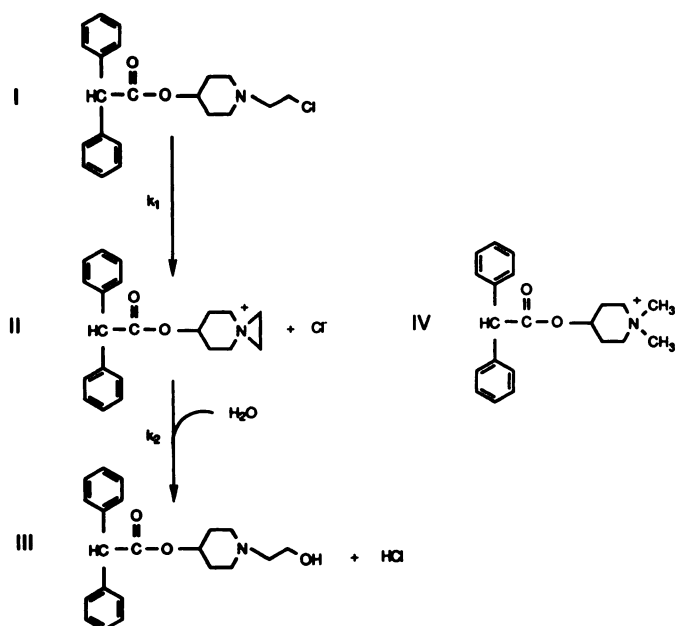


Fig. 1. Comparison of the structures of 4-DAMP mustard (I), its aziridinium ion (II), its alcoholic hydrolysis product (III), and 4-DAMP (IV). The rate constants for formation and decay of the aziridinium ion are denoted by  $k_1$  and  $k_2$ , respectively.

to have high affinity for the  $M_3$  muscarinic subtype, as well as the  $M_1$  type (11, 12). This paper describes an alternative synthesis of 4-DAMP mustard, its conversion to an aziridinium ion, and its muscarinic receptor-binding properties in rat brain, heart, and submaxillary gland. We conclude, from these results, that 4-DAMP mustard forms an aziridinium ion that binds irreversibly to muscarinic receptors, exhibits preferential alkylation of the  $M_3$  muscarinic subtype over the  $M_2$ , and is an ideal irreversibly alkylating agent.

## Materials and Methods

**Synthesis of 4-DAMP mustard.** *N*-Boc-4-piperidinol was synthesized from di-*tert*-butyl-dicarbonate and 4-piperidinol using a method analogous to that described by Jacobson and co-workers (13) for the synthesis of *t*-Boc derivatives of pyrrolidine. Di-*tert*-butyl dicarbonate (23 ml; 0.100 mol) was slowly added to a solution of 4-piperidinol (10.23 g; 0.101 mol) in 44 ml of methanol, at 0°. The solution was warmed to 25° and stirred at room temperature for 1 hr, after which the reaction was complete, as judged by TLC (chloroform/methanol, 1:1). Nearly all of the volatile material was removed under reduced pressure, to yield *N*-Boc-4-piperidinol as a yellow oil. The acid chloride of diphenylacetic acid was synthesized by refluxing a solution of diphenylacetic acid (21.43 g; 0.101 mol) in 70 ml of thionylchloride (0.96 mol) for 30 min. After removal of excess thionylchloride by distillation and reduced pressure, the resulting diphenylacetylchloride was slowly added to a stirred solution containing all of the *N*-Boc-4-piperidinol described above, 60 ml of chloroform, and 30 ml of pyridine, at 0°. The mixture was stirred at room temperature for 1 hr and refluxed for 30 min. Additional chloroform was added to the mixture, and the *N*-Boc-4-piperidinyl diphenylacetate product was extracted into the chloroform with 1 M  $K_2CO_3$ . The chloroform and some of the pyridine were removed under reduced pressure. The Boc group was cleaved from the ester by adding an excess of trifluoroacetic acid (60 ml) to a stirred solution of the *N*-Boc-4-piperidinyl diphenylacetate in 20 ml of chloroform, at 0°. The mixture was allowed to warm to room temperature and was stirred for 1 hr, after which deblocking was complete, as judged by TLC

(chloroform/methanol, 1:1). The reaction mixture was made basic with 1 M  $K_2CO_3$ , and the 4-piperidinyl diphenylacetate was extracted into chloroform. Chloroform and residual pyridine were removed under reduced pressure, to yield 30 g of crude 4-piperidinyl diphenylacetate. This material was dissolved in methanol, and a slight excess of HCl was added. All volatile material was removed under reduced pressure, and the hydrochloride salt was recrystallized twice from 2-butanone-ether, to yield 18.9 g (57% yield) of 4-piperidinyl diphenylacetate · HCl.

*N*-2-Hydroxyethyl-4-piperidinyl diphenylacetate was synthesized from 4-piperidinyl diphenylacetate and iodoethanol. The hydrochloride salt of 4-piperidinyl diphenylacetate (6.0 g; 18.1 mmol) was dissolved in water and extracted into chloroform with 1 M  $K_2CO_3$ . The chloroform was removed under reduced pressure. The resulting oil was dissolved in 116 ml of anhydrous ethanol, 31.8 g (185 mmol) of iodoethanol was added, and the mixture was allowed to reflux for 3 hr. TLC analysis (methanol) showed a relatively small amount of residual starting ester ( $R_f$  = 0.10), *N*-2-hydroxyethyl-4-piperidinyl diphenylacetate ( $R_f$  = 0.43), and a more polar product ( $R_f$  = 0.75) likely to be the *N,N*-diethanol quaternary salt of 4-piperidinyl diphenylacetate. The volatile material was removed under reduced pressure, and the residual oil was partially dissolved in ether. The polar contaminant was extracted into water with 1 M  $K_2CO_3$ , and the ether phase, containing *N*-2-hydroxyethyl-4-piperidinyl diphenylacetate, was dried with anhydrous  $MgSO_4$ . This mixture was then filtered into a solution of anhydrous ether containing an excess of oxalate. The resulting precipitate was partially dissolved in water, and *N*-2-hydroxyethyl-4-piperidinyl diphenylacetate was extracted into chloroform with 1 M  $K_2CO_3$ . The chloroform was removed under reduced pressure, and the residual oil, containing a mixture of *N*-2-hydroxyethyl-4-piperidinyl diphenylacetate and residual 4-piperidinyl diphenylacetate, was purified by column chromatography, using silica gel and methanol as the solvent, to yield 2.93 g (8.63 mmol; 48% yield) of *N*-2-hydroxyethyl-4-piperidinyl diphenylacetic acid as a clear oil.

The hydrochloride salt of 4-DAMP mustard was synthesized by chlorinating *N*-2-hydroxyethyl-4-piperidinyl diphenylacetate with 10-fold excess thionylchloride, essentially as described by Barlow *et al.* (10). This product had a melting point (136–137°) the same as that (136°) reported by Barlow *et al.* (10). The oxalate salt of 4-DAMP mustard was prepared by dissolving the hydrochloride salt in ice-cold water and rapidly extracting the free base into ice-cold ether with 1 M  $K_2CO_3$ . The ether extract was filtered through anhydrous  $MgSO_4$ , into a solution of oxalic acid in anhydrous ether. The resulting white precipitate had a melting point of 128.5–129.5°. The elemental composition of this material was determined by Galbraith Laboratories (Knoxville, TN) to be C, 61.22%; H, 5.94%; N, 2.99%; O, 22.47%; and Cl, 8.01%, which is in close agreement with the theoretical values for 4-DAMP mustard · oxalate (C, 61.7%; H, 5.90%; N, 3.13%; O, 21.4%; Cl, 7.92%). The oxalate salt of 4-DAMP mustard was recrystallized from methanol/ether.

### Measurements of formation and decay of the aziridinium ion.

The oxalate salt of 4-DAMP mustard was used in experiments where chloride release and the aziridinium ion were measured. A solution of 4-DAMP mustard (1 mM) was prepared by first dissolving the mustard in a volume of acetone equivalent to 40% of the final volume and then diluting the solution to final volume with 10 mM sodium-potassium phosphate buffer (pH 7.4). In experiments where the effect of pH on chloride release was investigated, a solution of 4-DAMP mustard was prepared in the same manner, except that a 10 mM citrate buffer was used. This buffer was titrated to the indicated pH values with Tris base.

The aziridinium ion was measured using the method described by Gill and Rang (1). Aliquots (5 ml) of the solution of 4-DAMP mustard (1.0 mM) were removed at various times, and cyclization was stopped by the addition of 1 ml of 0.2 M acetic acid. An aliquot (1.0 ml) of a solution of sodium thiosulfate (10 mM) was added, and the solution was allowed to stand for 20 min. Residual thiosulfate was estimated by

titration with a standardized solution of potassium triiodide (10 mM), using a starch solution as the indicator.

Chloride ion was measured at various times during cyclization, using the method of Volhard (14).

The rate constants for formation and decay of the aziridinium ion were estimated by nonlinear regression analysis, as described previously (4). The rate constant for formation of the aziridinium ion ( $k_1$ ) was estimated from the chloride release data by fitting the data to the following equation, by nonlinear regression analysis:

$$y = P \times (1 - e^{-k_1 t}) \quad (1)$$

In this equation,  $y$  denotes the chloride released,  $P$  denotes the starting amount of 4-DAMP mustard, and  $t$  denotes time. The measurements of the aziridinium ion concentration ( $a$ ) as a function of time were fitted to the following equation by nonlinear regression analysis, to estimate the rate constants for both formation ( $k_1$ ) and decay ( $k_2$ ) of the aziridinium ion:

$$a = P \times \frac{k_1}{k_2 - k_1} \times (e^{-k_1 t} - e^{-k_2 t}) \quad (2)$$

The  $pK_a$  of 4-DAMP mustard was estimated by measuring chloride release at various pH values and fitting the data to an equation similar to eq. 1, to obtain estimates of the apparent rate constant for formation of the aziridinium ion ( $k_{app}$ ). These  $k_{app}$  values were fitted to the following equation

$$k_{app} = \frac{k_1}{1 + 10^{(pK_a - pH)}} \quad (3)$$

in which  $k_1$  is the rate constant for chloride release from the free base and  $K_a$  is the acid dissociation constant of 4-DAMP mustard.

**Tissue preparation.** All tissues were obtained from male Sprague-Dawley rats weighing 200–275 g. Rats were euthanized with ether vapor in closed bell jars for 15–25 minutes, and forebrain (telencephalon and diencephalon), heart, and submaxillary glands were removed and placed in 0.9% saline. Tissues were homogenized with a Polytron (Brinkman Instruments, Westbury, NY) at setting 5, in 20 volumes of buffer containing 30 mM Na/HEPES, pH 7.5, 100 mM NaCl, and 0.5 mM EGTA. Homogenates were centrifuged at  $30,000 \times g$  for 10 min, supernatants were discarded, and pellets were resuspended to a concentration of 25 mg (heart and submaxillary gland) or 50 mg (brain) of original wet weight of tissue per milliliter of buffer.

In order to determine the irreversible effects of 4-DAMP mustard on the dissociation constant ( $K_d$ ) and binding capacity ( $B_{max}$ ) of [ $^3H$ ] NMS for muscarinic receptors, tissues were first treated with 4-DAMP mustard and then assayed for ligand binding. Aliquots of homogenates were pipetted into plastic tubes and preincubated at  $37^\circ$  for 10 min. A solution of 4-DAMP mustard that had been incubated at  $37^\circ$  for 30 min, to allow formation of the aziridinium ion, was added at a final concentration of 9 nM, with respect to the parent mustard, and tissues were incubated further for various times. Control homogenates were prepared in the same manner, except for exposure to 4-DAMP mustard during the incubation period. Sodium thiosulfate (0.01 M) was added to the tubes to stop the reaction, and the incubation was continued for 10 more minutes before centrifugation at  $30,000 \times g$  for 10 min. Tissues were washed extensively three times, to remove thiosulfate, excess 4-DAMP mustard, and its transformation products, by centrifugation at  $30,000 \times g$  for 10 min, followed by resuspension in 30 ml of HEPES buffer. The resulting pellet was frozen immediately.

In experiments where the rates of alkylation by 4-DAMP mustard were measured, the reaction between homogenates and 4-DAMP mustard was stopped immediately by the addition of 0.1 mM atropine and 0.01 M sodium thiosulfate, and pellets were washed four times as described above.

**Binding assays.** The binding of the specific muscarinic antagonist [ $^3H$ ]NMS (73.1 Ci/mmol; DuPont-New England Nuclear, Boston, MA) was measured as described previously (15). Homogenates were incu-

bated at  $25^\circ$  with various concentrations of [ $^3H$ ]NMS, for 30 min (brain and heart) or 60 min (submaxillary gland), in a final volume of 1 ml. All assays were run in triplicate. Membrane-bound radioactivity was trapped by vacuum filtration, using a cell harvester (Brandel, Gaithersburg, MD), on glass fiber filters (GF/B Whatman Inc, Clifton NJ) that had been presoaked with 0.1% polyethylenimine HCl, pH 7.0. Filters were washed with four 4-ml aliquots of ice-cold 0.9% saline. Binding in the presence of 10  $\mu$ M atropine was defined as nonspecific.

In competitive binding experiments, a single concentration of [ $^3H$ ] NMS (0.5 nM) was used, and various concentrations of nonlabeled drugs, including 4-DAMP, 4-DAMP mustard, and its transformation products, were included in the binding assays during the incubation period. The  $K_d$  and  $B_{max}$  of [ $^3H$ ]NMS in the various tissues were calculated as described previously (15).

The observed rate constant ( $k_{obs}$ ) of the aziridinium ion of 4-DAMP mustard for alkylation of muscarinic receptors was estimated by incubating tissue homogenates with a given concentration of 4-DAMP mustard for various times, washing the tissue, and then measuring the binding of [ $^3H$ ]NMS at a single concentration (0.5 nM). The resulting data were fitted to the following equation by nonlinear regression analysis:

$$B = e^{-k_{obs} t}$$

in which  $B$  denotes the fractional specific binding of [ $^3H$ ]NMS and  $t$  denotes time.

**Isolated ileum.** The whole ileum was mounted longitudinally in an organ bath containing KRB buffer (124 mM NaCl, 5 mM KCl, 1.3 mM  $MgCl_2$ , 26 mM  $NaHCO_3$ , 1.2 mM  $KH_2PO_4$ , 1.8 mM  $CaCl_2$ , 10 mM glucose), at  $37^\circ$ , that was aerated continually with  $O_2/CO_2$  (19:1). Isometric contractions were measured with a force displacement transducer and polygraph. The ileum was equilibrated for 40 min at a resting tension of 0.5 g. Three test doses of oxotremorine-M were added to ensure reproducibility of the preparation. To calculate an  $EC_{50}$  value for oxotremorine-M, five to seven concentrations of the compound (spaced geometrically every 0.33 log units) were added cumulatively to the bath, and contractile responses were measured. Tissues were then incubated with the aziridinium ion of 4-DAMP mustard for 90 min. KRB buffer and drug solutions were changed every 30 min during the incubation period. The tissue was then washed two times with fresh KRB buffer and incubated for an additional 15 min with 0.01 M  $Na_2SO_3$ , to inactivate the aziridinium ion. The tissue was again washed twice and was rested for 15 min before a second dose-response experiment was performed. Washing procedures were repeated, and a third dose-response assay was performed after 1 hr.

## Results

**Formation and decomposition of the aziridinium ion.** 4-DAMP mustard goes through two sequential reactions in aqueous solution at neutral pH, as outlined in Fig. 1. The first of these is the formation of the cyclic aziridinium ion, and the second is the hydrolysis of the aziridinium ion to the alcohol. The first reaction occurs with the simultaneous release of an equivalent amount of chloride, which provides a convenient means to follow this reaction. Fig. 2 shows the release of chloride from a solution of 4-DAMP mustard (1.0 mM) at pH 7.4. The release of chloride at  $37^\circ$  and  $22^\circ$  resembled a first-order process, having half-times of 5.77 and 23.1 min, respectively. The rate of release of chloride at  $0^\circ$  was very slow; after 24 hr only 60% of the total amount of chloride was released. Table 1 lists the estimates of the rate constants for formation of the aziridinium ion from the chloride release data.

We also directly measured the concentration of the aziridinium ion at various times. These data provided a means of estimating both the rate of formation and the rate of decay of the aziridinium ion. Fig. 3 shows the concentration of the

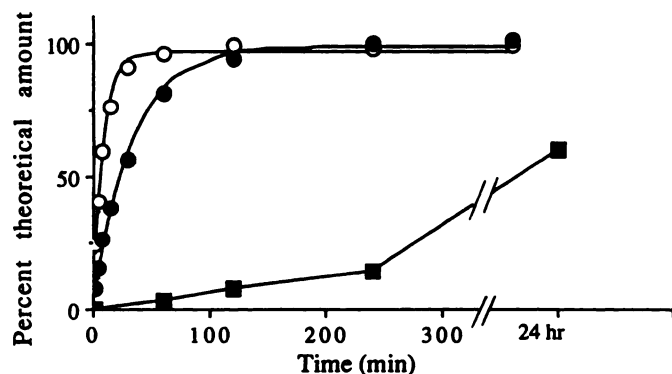


Fig. 2. Chloride release from 4-DAMP mustard in aqueous solution (pH 7.4) at 37° (○), 25° (●), and 0° (■). Data represent the means of two separate experiments. The theoretical curves represent the least squares fit of the data to eq. 1.

TABLE 1

Rate constants for the formation ( $k_1$ ) and decay ( $k_2$ ) of the aziridinium ion

Values were estimated from the data in Figs. 2 and 3.

	Chloride release, $k_1$ $\text{min}^{-1}$	Aziridinium ion	
		$k_1$ $\text{min}^{-1}$	$k_2$ $\text{min}^{-1}$
25°	$0.030 \pm 0.002$	$0.034 \pm 0.001$	$0.002 \pm 0.0001$
37°	$0.122 \pm 0.013$	$0.093 \pm 0.011$	$0.008 \pm 0.003$

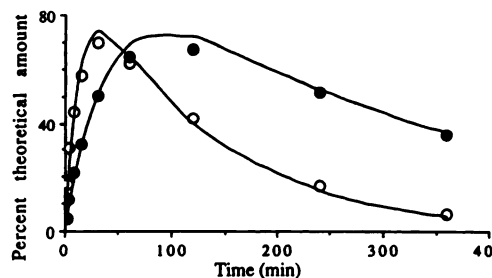


Fig. 3. Formation and decay of the aziridinium ion of 4-DAMP mustard in aqueous solution (pH 7.4) at 37° (○) and 25° (●). Data points at 37° are mean values from three experiments, and data at 25° represent the means of two separate experiments. The theoretical curves represent the least squares fit of the data to eq. 2.

aziridinium ion in a solution of 4-DAMP mustard (1.0 mM) at pH 7.4. The aziridinium ion reached a maximal concentration (75%) after 32 min at 37° and after 90 min (73%) at 22°. Table 1 gives the rate constants for the formation and decay of the aziridinium ion, as estimated from the data in Fig. 3. It can be seen that there is general agreement (within 2-fold) between the estimates of the rate constant for cyclization measured from the chloride release data and from the aziridinium ion data.

The rate of formation of the aziridinium ion was dependent on pH, as shown in Fig. 4. At low pH cyclization was very slow, whereas at high pH the rate of cyclization approached a maximal plateau. The data are generally consistent with the hypothesis that the rate of cyclization is proportional to the relative amount of 4-DAMP mustard in the unprotonated state. This model predicts that the observed rate constant for cyclization should increase as a mass action-like function of the pH, with the half-maximal rate occurring at a pH equivalent to the  $pK_a$  of 4-DAMP mustard. Using nonlinear regression analysis,

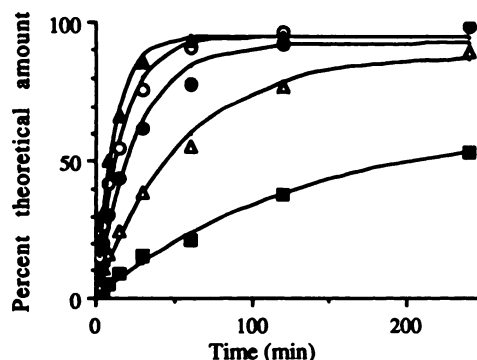
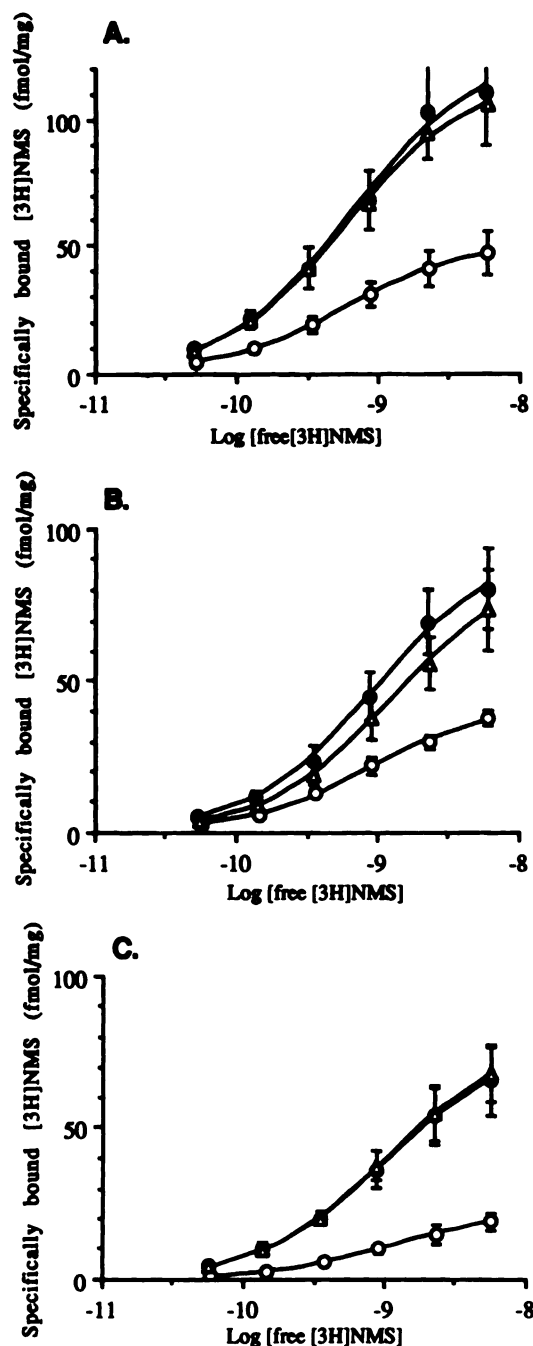


Fig. 4. Effects of pH on the rate of chloride release from 4-DAMP mustard. Chloride release was determined at pH 4 (■), 5 (△), 6 (●), 7 (○), and 8 (▲). Data points represent the means of two separate experiments, and the variability between measurements was always <5%. The theoretical curves represent the least squares fit of the data to eq. 1.

we fitted eq. 3 to the data and obtained an estimate of 5.96 for the  $pK_a$  of 4-DAMP mustard. This value is similar to that reported for other 2-chloroethylamines (16). It can be estimated that at pH 7.4, 96.5% of 4-DAMP mustard is in the unprotonated state, which accounts, in part, for its low solubility at neutral pH.

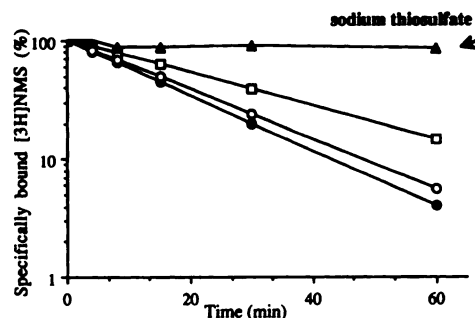
**Muscarinic receptor binding activity.** When homogenates of forebrain, heart, and submaxillary gland were exposed to 4-DAMP mustard, an irreversible inhibition of [ $^3\text{H}$ ]NMS binding was observed. Tissues were incubated with the aziridinium ion of 4-DAMP mustard (9 nM) for 1 hr, washed extensively, and then assayed for the specific binding of [ $^3\text{H}$ ]NMS at various concentrations of the radioligand. Control homogenates were prepared identically, except for exposure to the mustard, and in protection experiments  $10^{-6}$  M atropine was allowed to equilibrate with the preparation for 10 min before the addition of the 4-DAMP mustard (Fig. 5). The results were examined by nonlinear regression analysis, to determine the effect of incubation with the aziridinium ion on the dissociation constants and binding capacities of [ $^3\text{H}$ ]NMS in the three tissues. Control binding was consistent with a single-site model, with dissociation constants of 0.64, 1.02, and 0.95 nM for the forebrain, heart, and submaxillary gland, respectively. Pretreatment with the aziridinium ion resulted in a 56% reduction in the binding capacities of the brain and heart and a 71% reduction in that of the gland, without significantly affecting the  $K_d$ . As described below, it was possible to achieve an even more selective alkylation of muscarinic receptors in the submaxillary gland, compared with the heart, by using more favorable concentrations of tissue and 4-DAMP mustard. Our estimates of the dissociation constants of [ $^3\text{H}$ ]NMS are approximately 2-fold greater than those typically measured for [ $^3\text{H}$ ]NMS in previous studies, using a buffer system similar to that used here (17). We have no adequate explanation for this difference.

Experiments were performed to assess the effects of tissue concentration and temperature on the rate of alkylation of muscarinic receptors by 4-DAMP mustard. In these experiments, brain homogenates were incubated with the aziridinium ion of 4-DAMP mustard (94 nM) for various times, washed extensively, and then assayed for [ $^3\text{H}$ ]NMS binding at a single concentration of 0.5 nM. The results in Fig. 6 show that the alkylation of muscarinic receptors by the aziridinium ion of 4-DAMP mustard resembles a simple first-order process, as in-

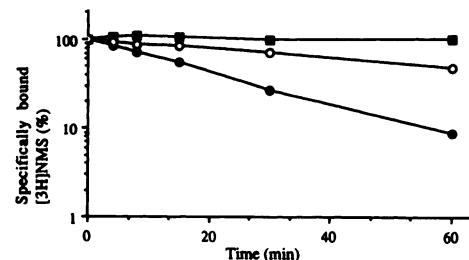


**Fig. 5.** Effects of 4-DAMP mustard pretreatment on [ $^3\text{H}$ ]NMS binding in rat forebrain (A), heart (B), and submaxillary gland (C). Specific binding of [ $^3\text{H}$ ]NMS was measured in homogenates that had been incubated for 60 min at 37° with the aziridinium ion of 4-DAMP mustard ( $10^{-8}$  M) and washed extensively (○). In protection experiments, atropine ( $10^{-6}$  M) was incubated with the homogenates for 10 min before the addition of the aziridinium ion (Δ). ●, Control homogenates. Data points represent the mean binding value of three experiments, each done in triplicate. The theoretical curves represent the least squares fit of the data to a one-site model.

indicated by the linear plot of the logarithm of the number of unalkylated receptors versus time. Fig. 6 also shows that the half-time of receptor alkylation was approximately 13.5 min at tissue concentrations between 5 and 10 mg (original wet tissue weight)/ml and 25 min at a concentration of 25 mg/ml. These results suggest that high tissue concentrations ( $>10$  mg/ml)



**Fig. 6.** Effect of tissue concentration and sodium thiosulfate (0.01 M) on the rate of alkylation of [ $^3\text{H}$ ]NMS binding sites by 4-DAMP mustard. Homogenates of rat forebrain were incubated with the aziridinium ion of 4-DAMP mustard ( $10^{-7}$  M) at 37° for various times, at tissue concentrations of 5 mg/ml (●), 10 mg/ml (○), and 25 mg/ml (□). Some homogenates were incubated with 4-DAMP mustard that had been treated with thiosulfate (Δ). Tissues were washed extensively and then assayed for specific binding of [ $^3\text{H}$ ]NMS at 37°. The concentration of [ $^3\text{H}$ ]NMS was 0.5 nM. Data points represent the mean binding value of three experiments, each done in triplicate.



**Fig. 7.** Effects of temperature on the rate of alkylation of [ $^3\text{H}$ ]NMS binding sites by 4-DAMP mustard. Brain homogenates were pretreated with the aziridinium ion of 4-DAMP mustard ( $10^{-7}$  M) for the indicated times, at temperatures of 0° (■), 22° (○), and 37° (●), washed extensively, and then assayed for specific binding of [ $^3\text{H}$ ]NMS. The concentration of [ $^3\text{H}$ ]NMS was 0.5 nM. Data points represent the mean binding value of three experiments, each done in triplicate.

may deplete the aziridinium ion concentration. No alkylation of muscarinic receptors by 4-DAMP mustard (94 nM) was observed when a solution of the aziridinium ion was treated with sodium thiosulfate (10 mM) for 10 min at 37° before addition to the brain homogenate (Fig. 6).

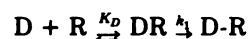
The rate of receptor alkylation by 4-DAMP mustard was temperature sensitive. Brain homogenates were pretreated with the aziridinium ion of 4-DAMP mustard (94 nM) for various times and at varying temperatures. Tissues were washed extensively and assayed for specific [ $^3\text{H}$ ]NMS binding as described above. A maximal rate of alkylation occurred at 37°, whereas no significant alkylation was observed at 0° (Fig. 7). Slight alkylation occurred at higher concentrations of 4-DAMP mustard at 0°, with a maximum of 18% alkylation after 1 hr at a concentration of 94  $\mu\text{M}$  (data not shown). The half-times for alkylation at 37° and 25° were 16.5 and 60.3 min, respectively.

At neutral pH, a solution of 4-DAMP mustard contains a mixture of compounds with potentially different pharmacological properties. The dissociation constants of 4-DAMP mustard and its transformation products for muscarinic receptors were determined by measuring their ability to inhibit [ $^3\text{H}$ ]NMS binding competitively. Due to the negligible rate at which the aziridinium ion of 4-DAMP mustard inactivated muscarinic binding sites at low temperatures, the affinity of this species could be estimated by using standard competitive binding techniques at 0°. A solution of 4-DAMP mustard was preincubated

at 37° for 30 min, to allow formation of the aziridinium ion, and various concentrations of this solution were added to the competitive binding assay at 0°. Previous reports have shown that equilibrium in brain homogenates was reached after 1 hr at 0° (15); thus, brain as well as heart and gland preparations were incubated for a 1-hr period. Fig. 8 shows that the aziridinium ion had a 2-fold lower affinity in brain, heart, and submaxillary gland (9.9, 70, and 10.5 nM, respectively), compared with the reversible muscarinic antagonist 4-DAMP (4.97, 37.6, and 5.14 nM for the brain, heart, and gland, respectively). The affinity values for the former are based on the assumption that the aziridinium ion represents approximately 70% of the initial concentration of the parent mustard (see above). The affinities of the parent mustard and alcoholic hydrolysis product were also determined at 0° (Fig. 8). The parent compound

(4-DAMP mustard) was 30–50-fold less potent than the aziridinium ion, with  $K_d$  values of 0.36, 1.82, and 0.52  $\mu\text{M}$  for the brain, heart, and submaxillary glands, respectively. The alcoholic hydrolysis product had higher affinity in the three tissues, with  $K_d$  values of 80, 469, and 46 nM, respectively.

**Kinetic analysis.** The simplest scheme for the interaction of the aziridinium ion of 4-DAMP mustard (D) with the muscarinic receptor (R) is the following:



in which the aziridinium ion rapidly forms a reversible complex with the muscarinic receptor (DR), which converts to a covalent complex (D-R) at a relatively slower rate. This model predicts that the rate of alkylation of muscarinic receptors by 4-DAMP mustard should increase as a mass action-like function of the aziridinium ion concentration. Also, the observed rate constant for alkylation ( $k_{\text{obs}}$ ) should approach a maximum value equivalent to  $k_1$  at high concentrations of the aziridinium ion, and the half-maximal rate of alkylation should occur at an aziridinium ion concentration equivalent to the dissociation constant ( $K_d$ ) of the reversible complex (DR). Thus, the overall rate of alkylation depends on both the affinity of the aziridinium ion for the muscarinic receptor and the rate constant for alkylation.

To estimate the kinetic parameters of 4-DAMP mustard in the heart and submaxillary gland, the rate of alkylation was measured at various concentrations of the aziridinium ion. Homogenates of the heart and submaxillary gland were incubated with increasing concentrations of the aziridinium ion of 4-DAMP mustard, washed extensively, and assayed for [ $^3\text{H}$ ] NMS binding. The results in Fig. 9 show that the loss of [ $^3\text{H}$ ] NMS binding sites in the gland (Fig. 9A) and heart (Fig. 9B) in the presence of the aziridinium ion resembled a single first-order process up to about 90% receptor alkylation but that some deviation from the first-order model was noted at 60 min in the presence of the highest concentrations of 4-DAMP mustard (0.2 and 1  $\mu\text{M}$ ). The observed rate constants ( $k_{\text{obs}}$ ) for alkylation were calculated from the data as described in Materials and Methods, and these values are plotted, in Fig. 10, versus the concentration of the aziridinium ion. It can be seen that the  $k_{\text{obs}}$  increases as a mass action-like function of the aziridinium ion concentration in both the heart and submaxillary gland and that the potency of 4-DAMP mustard is about 6.3-fold greater in the gland. Nonlinear regression analysis of the data in Fig. 10, according to the Michaelis-Menten equation, yielded similar maximal rate constants for alkylation ( $k_1$ ) in the gland (0.090  $\text{min}^{-1}$ ) and heart (0.116  $\text{min}^{-1}$ ) and a 6.3-fold lower  $K_d$  value in the gland (7.2 nM), compared with the heart (46 nM). These kinetic  $K_d$  values are in general agreement with those measured in competition experiments with [ $^3\text{H}$ ] NMS at 0° (see Table 2).

In order to achieve a more selective alkylation of muscarinic receptors in the submaxillary gland, the kinetic experiments were repeated in the presence of the cardioselective muscarinic antagonist AF-DX 116 (1.0  $\mu\text{M}$ ) (see Fig. 9, C and D). The results in Fig. 10 show that, in terms of potency, the aziridinium ion exhibits about 24-fold greater selectivity for muscarinic receptors in the submaxillary gland, compared with the heart, in the presence of 1.0  $\mu\text{M}$  AF-DX 116. In the presence of AF-DX 116, the observed  $K_d$  values of the aziridinium ion were 41 and 980 nM in the gland and heart, respectively. The results in Fig. 10 provide no evidence for an effect of AF-DX 116 on the

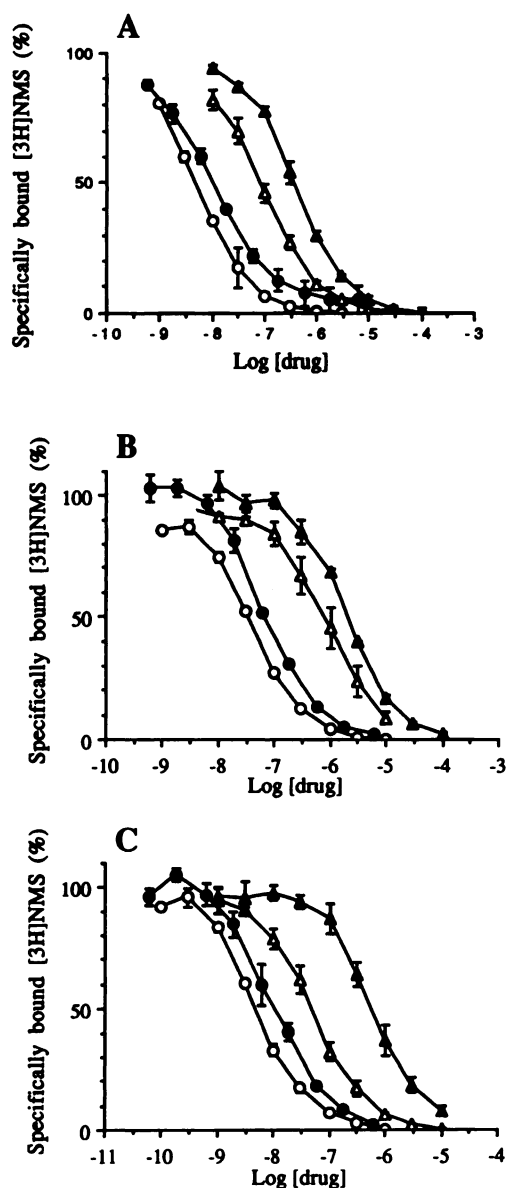
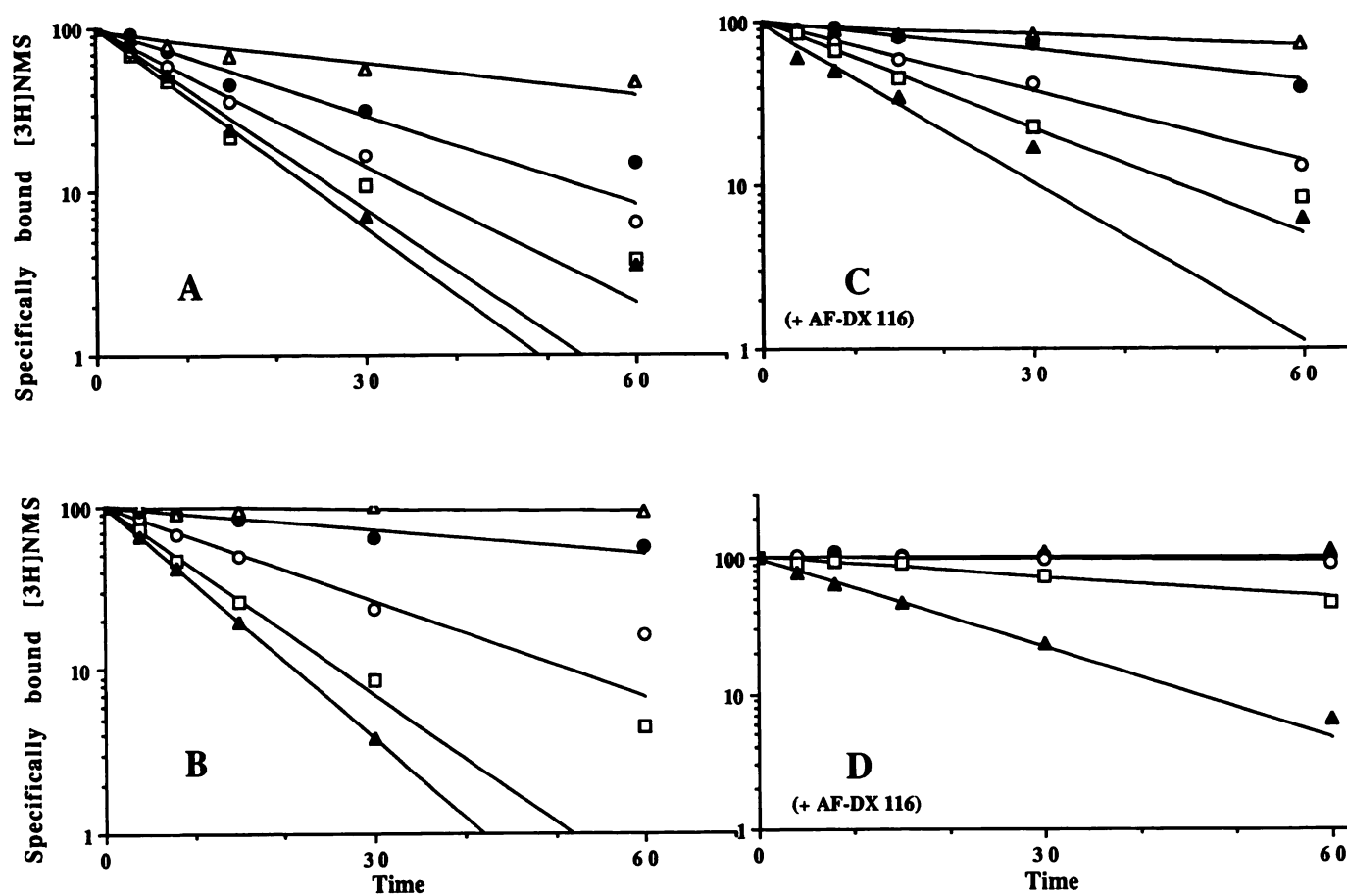
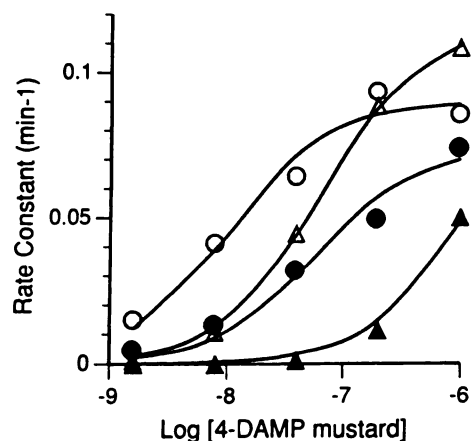


Fig. 8. Competitive inhibition of [ $^3\text{H}$ ]NMS binding in rat forebrain (A), heart (B), and submaxillary gland (C) by 4-DAMP (○), 4-DAMP mustard (▲), its aziridinium ion (●), and its alcoholic hydrolysis product (△). Specific binding of [ $^3\text{H}$ ]NMS was measured at 0° in the presence of the indicated concentrations of the various compounds. The concentration of [ $^3\text{H}$ ]NMS was 0.5 nM. All experiments were run three times, each done in triplicate.



**Fig. 9.** Kinetics of the alkylation of  $[^3\text{H}]\text{NMS}$  binding sites by 4-DAMP mustard (A and B), and the effect of AF-DX 116 on these rates (C and D), in the rat submaxillary gland (A and C) and heart (B and D). Homogenates of rat gland and heart were incubated at  $37^\circ$  with 4-DAMP mustard for the indicated times, at concentrations of 1.6 nM ( $\Delta$ ), 8 nM ( $\bullet$ ), 40 nM ( $\circ$ ), 200 nM ( $\square$ ), and 1  $\mu\text{M}$  ( $\blacktriangle$ ). AF-DX 116 (1  $\mu\text{M}$ ) was added to the appropriate tubes 10 min before the addition of 4-DAMP mustard. Homogenates were then washed extensively and assayed for specific binding of  $[^3\text{H}]\text{NMS}$  at a single concentration of 0.5 nM. Each point represents the mean binding values of five experiments, each done in triplicate.



**Fig. 10.** Plot of the observed rate constants versus the logarithm of concentrations of 4-DAMP mustard in the submaxillary gland (circles) and heart (triangles), alone (open symbols) and in the presence of AF-DX 116 (closed symbols). The observed rate constants were determined from the data in Fig. 9.

maximal rate constant for alkylation ( $k_1$ ) in either the gland or heart.

The kinetic analysis shown in Fig. 10 predicts that it should be possible to alkylate muscarinic receptors in the gland selectively, using a relatively low concentration of the aziridinium

**TABLE 2**

**Dissociation constants of 4-DAMP, 4-DAMP mustard, and its transformation products in rat forebrain, heart, and submaxillary gland**

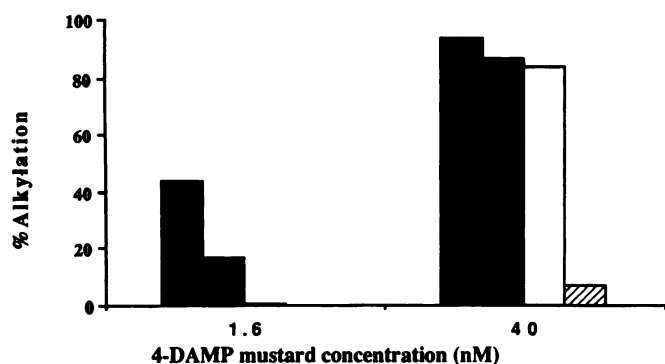
Values (mean  $\pm$  standard error) were estimated from the data shown in Fig. 8 and are expressed in  $\text{pK}_D$  units ( $-\log K_D$ ).

Compound	$\text{pK}_D$		
	Forebrain	Heart	Submaxillary gland
4-DAMP	$8.30 \pm 0.05$	$7.43 \pm 0.06$	$8.29 \pm 0.05$
4-DAMP mustard	$6.44 \pm 0.12$	$5.76 \pm 0.05$	$6.26 \pm 0.19$
Aziridinium ion	$8.09 \pm 0.17$	$7.21 \pm 0.04$	$8.04 \pm 0.13$
		(7.34) <sup>a</sup>	(8.14) <sup>a</sup>
Alcoholic hydrolysis product	$7.10 \pm 0.16$	$6.09 \pm 0.31$	$7.31 \pm 0.13$

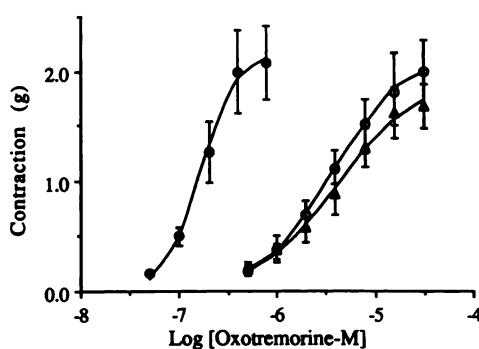
<sup>a</sup> Values obtained from kinetic analysis shown in Fig. 10.

ion for a long time. Accordingly, Fig. 11 shows that, at a concentration of 1.6 nM, the aziridinium ion caused virtually no alkylation of muscarinic receptors in the heart, after 30 min, but eliminated 48% of the receptors in the gland. In the presence of 1.0  $\mu\text{M}$  AF-DX 116, the aziridinium ion (40 nM) caused 86% alkylation of muscarinic receptors in the gland after 1 hr but only 7% alkylation of receptors in the heart.

**Isolated rat ileum.** Treatment of the isolated rat ileum with 4-DAMP mustard caused an irreversible blockade of contractions elicited by the muscarinic agonist oxotremorine-M, and



**Fig. 11.** Selected conditions of receptor alkylation by 4-DAMP mustard alone in the submaxillary gland (■) and heart (□) and in the presence of AF-DX 116 (□, gland; ▨, heart). The percentages of receptor alkylation were calculated from the data in Fig. 9. Incubation times were 30 and 60 min at concentrations of 1.6 and 40 nM, respectively.



**Fig. 12.** Effects of 4-DAMP mustard on the dose-response curve of oxotremorine-M in the isolated rat ileum. Oxotremorine-M-mediated contractions were first measured in control rat ilea (●). Then the ilea were incubated with 4-DAMP mustard ( $10^{-6}$  M) for 90 min and washed extensively, and additional dose-response curves were run immediately (○) and after 90 min (▲). Data points represent the means of three experiments.

this blockade persisted after extensive washing. Oxotremorine-M contracted the ileum with an  $EC_{50}$  value of approximately  $0.15 \mu\text{M}$ . Treatment with 4-DAMP mustard (9 nM, for 90 min) caused a 27-fold rightward shift of the concentration-effect curve, with an accompanying slight decrease in the maximal contraction (Fig. 12). This 27-fold shift in the oxotremorine-M concentration-effect curve was still maintained 90 min later (Fig. 12), indicating that the inhibitory effect of 4-DAMP mustard was irreversible and that 4-DAMP mustard could not be washed off the receptor.

## Discussion

In experiments where forebrain, heart, and submaxillary gland were incubated with 4-DAMP mustard, a decrease in maximal binding of  $[^3\text{H}]\text{NMS}$ , without an accompanying change in  $K_d$ , was observed. This action was prevented by the addition of atropine during the incubation, which is consistent with the postulate that the aziridinium ion alkylates the recognition site of the muscarinic receptor. Previous reports on nitrogen mustard analogues of oxotremorine and benzilylcholine have shown that the pharmacological activity of these compounds can be attributed to their aziridinium ions (1, 3). Observations in this paper support the assumption that the aziridinium ion is also mainly responsible for the muscarinic antagonist actions of 4-DAMP mustard. Incubation of the

aziridinium ion with thiosulfate prevented alkylation of the receptor. It is known that thiosulfate reacts covalently with aziridinium ions to inactivate them; thus, these results imply that the aziridinium ion is necessary for alkylation of the recognition site. In addition, the affinity of the aziridinium ion, measured at  $0^\circ$  without covalent bond formation at the recognition site, was higher than both that of the parent mustard and that of the alcoholic hydrolysis product, indicating that this cyclized species is the most potent form, therefore accounting for the antimuscarinic activity. For a variety of other tertiary amine muscarinic antagonists, it has been demonstrated that the antimuscarinic activity resides in the protonated species (18–21). Consequently, the inactivity of the parent mustard might seem to be due to its weak base strength ( $pK_a = 5.96$ ), resulting in a low degree of ionization (3.5%) at physiological pH. However, we have found that the  $IC_{50}$  value of the parent mustard is unaffected when the pH is reduced to 5.5 (data not shown), indicating that both the ionized and unionized forms are inactive. Thus, the inactivity of the parent mustard cannot be attributed to its weak base strength.

Although other nitrogen mustard derivatives, such as benzilylcholine mustard, acetylcholine mustard, and the oxotremorine nitrogen mustard derivatives, have been made, 4-DAMP mustard offers advantages over these as irreversible alkylators. Receptor alkylation by the aforementioned derivatives begins as a first-order process but reaches a plateau at submaximal levels (70%) (15, 16). Alternatively, 4-DAMP mustard can cause almost complete alkylation (97%) of muscarinic receptors, according to a single first-order process. 4-DAMP mustard also remains covalently bound to the receptor longer than do other nitrogen mustard compounds, such as benzilylcholine mustard. In the isolated ileum, 4-DAMP mustard caused a blockade of contractions that persisted after 90 min and extensive washing. In contrast, there is some recovery after 1 hr from the blockade produced by BCM, using similar washout conditions, in the isolated guinea pig ileum (22).

Another advantage to 4-DAMP mustard is its apparent selectivity. Because 4-DAMP mustard was derived from the selective antagonist 4-DAMP, it might be expected that this nitrogen mustard analogue would retain some of the selectivity exhibited by 4-DAMP itself. The tissues used in these experiments have different compositions of muscarinic receptor subtypes. The rat forebrain contains mainly the  $M_1$  subtype, based on its high affinity for pirenzepine, but also contains receptors of the  $M_3$  and  $M_4$  subtypes (23, 24). The  $M_2$  muscarinic receptors, characterized by their high affinity for AF-DX 116, account for most, if not all, of the binding sites in the mammalian heart (25, 26). The submaxillary gland has primarily  $M_3$  subtype receptors, with a small population of the  $M_1$  subtype, and both of these receptors have high affinity for 4-DAMP (11, 27, 28). These tissues provide the basis for exploring muscarinic subtype selectivity. In competitive binding experiments at  $0^\circ$ , the aziridinium ion of 4-DAMP mustard showed a selectivity profile similar to that of 4-DAMP,  $M_3 \approx M_1 > M_2$ .

Kinetic analysis shows that the overall rate of receptor alkylation by 4-DAMP mustard depends on the concentration of 4-DAMP mustard, its affinity for the receptor, and its rate constant of alkylation. Once it occupies the muscarinic receptor, the aziridinium ion alkylates  $M_2$  and  $M_3$  receptors at approximately the same rate, as indicated by the similar values for the rate constants for alkylation ( $k_1$ ) at these two sites.

Accordingly, similar rates of alkylation were observed in the submaxillary gland and heart when high concentrations of 4-DAMP mustard were used. However, selective alkylation of the M<sub>3</sub> muscarinic receptor was achieved using low concentrations of 4-DAMP mustard for relatively long periods of time. In the presence of AF-DX 116, 4-DAMP mustard shows enhanced selectivity towards the M<sub>3</sub> subtype. This can be useful, because AF-DX 116 is a reversible M<sub>2</sub> antagonist that can be washed off the receptor, leaving the M<sub>3</sub> receptors irreversibly blocked.

Previous pharmacological experiments on isolated tissues have shown that 4-DAMP mustard prevents responses elicited by M<sub>3</sub> muscarinic receptors selectively (10). Barlow *et al.* (10) reported much higher dose ratios for the blockade of M<sub>3</sub>-mediated contractions in the guinea pig ileum than for M<sub>2</sub>-mediated cardiac responses. In their experiments, the overall observed blockade of M<sub>3</sub>-mediated contractions was a combination of irreversible and reversible binding of 4-DAMP mustard to the muscarinic receptor. When expressed as a dose ratio, the irreversible component of blockade in the guinea pig ileum, after 90-min treatment with 4-DAMP mustard (10 nM) at 37°, varied between 14- and 90-fold. Our results showed that pretreatment of the rat ileum with the same concentration of 4-DAMP mustard caused a 27-fold shift in the oxotremorine-M dose-response curve. Thus, there is general agreement between our observations and those reported by Barlow *et al.* (10).

The results from our kinetic experiments in the heart and gland allow us to predict the irreversible effects of 4-DAMP mustard on various tissues, by determining the fraction of receptors occupied by 4-DAMP mustard at a given concentration. Based on the percentage of occupancy, dose ratios for antagonist effects can be calculated using the relationship described by Paton (29):

$$\% \text{ of occupancy} = \frac{(\text{dose ratio} - 1) \times 100}{\text{dose ratio}}$$

Using the kinetic parameters measured in the submaxillary gland, we predict that, at a concentration of 10 nM, 4-DAMP mustard would cause a 52-fold blockade of M<sub>3</sub>-mediated contractions in the isolated ileum after 90 min. This correlates with our observed dose ratio (27-fold) and those reported by Barlow *et al.* (10). Barlow and co-workers also showed that 4-DAMP mustard (10 nM, for 90 min) caused only a 2-fold shift of carbachol-mediated inhibition of contraction in the isolated guinea pig atria at 30° (10). At 37°, we predict a dose ratio of 4 for cardiac responses. These values are in good agreement, considering that greater blockade by 4-DAMP mustard occurs at 37° than at 30° (10) and that the rate of alkylation of muscarinic receptors decreases 3.7-fold as the temperature decreases from 37° to 25° (see Fig. 7).

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#### References

- 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).
- Young, J. M., R. Hiley, and A. S. V. Burgen. Homologues of benzilylcholine mustard. *J. Pharm. Pharmacol.* **24**: 950-954 (1972).
- 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).
- Ringdahl, B., B. Resul, F. J. Ehler, D. J. Jenden, and R. Dahlbom. The conversion of 2-chloroalkylamine analogues of oxotremorine to aziridinium ions and their interactions with muscarinic receptors in the guinea pig ileum. *Mol. Pharmacol.* **26**:170-179 (1984).
- Mei, L., W. R. Roeske, and H. L. Yamamura. Molecular pharmacology of muscarinic receptor heterogeneity. *Life Sci.* **45**:1831-1851 (1989).
- Akiba, I., T. Kubo, A. Maeda, H. Bujo, J. Nakai, M. Mishina, and S. Numa. Primary structure of porcine muscarinic acetylcholine receptor III and antagonist binding studies. *FEBS Lett.* **235**:257-261 (1988).
- Brann, M. R., N. J. Buckley, and T. I. Bonner. The striatum and cerebral cortex express different muscarinic receptor mRNAs. *FEBS Lett.* **230**:90-94 (1988).
- Maeda, A., T. Kubo, M. Mishina, and S. Numa. Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes. *FEBS Lett.* **239**:339-342 (1988).
- Buckley, N. J., T. I. Bonner, C. M. Buckley, and M. R. Brann. Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.* **35**:469-476 (1989).
- Barlow, R. B., M. K. Shepherd, and M. A. Veale. Some differential effects of 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine hydrochloride on guinea-pig atria and ileum. *J. Pharm. Pharmacol.* **42**:412-418 (1990).
- Lambrecht, G., R. Feifel, U. Moser, M. Wagner-Roder, L. K. Choo, J. Camus, M. Tastenoy, M. Waelbroek, C. Strohmman, R. Tacke, J. F. Rodrigues De Miranda, J. Christophe, and E. Mutschler. Pharmacology of hexahydrodifendol, hexahydrodiladifenidol and related selective muscarinic antagonists. *Trends Pharmacol. Sci.* (suppl.) **61**-64 (1989).
- Barlow, R. B., K. J. Berry, P. A. M. Glenton, N. M. Nikolaou, and K. S. Soh. A comparison of affinity constants for muscarinic-sensitive acetylcholine receptors in guinea pig atrial pace-maker cells at 29°C and in ileum at 29°C and 37°C. *J. Pharm. Pharmacol.* **58**:613-620 (1976).
- Bradbury, B. J., J. Baumgold, and K. A. Jacobson. Functionalized congener approach for the design of novel muscarinic agents: synthesis and pharmacological evaluation of N-methyl-N-[4-(1-pyrrolidinyl)-2-butyryl]amides. *J. Med. Chem.* **33**:741-748 (1990).
- Skog, D. A., and P. M. West. *Analytical Chemistry*. Holt, Rinehart and Winston, New York, 218-220 (1965).
- Ehler, F. J., and L. P. P. Tran. Regional distribution of M1, M2, and non-M1, non-M2 subtypes of muscarinic binding sites in rat brain. *J. Pharmacol. Exp. Ther.* **255**:1148-1157 (1990).
- Ehler, F. J., and D. J. Jenden. The binding of a 2-chloroethylamine derivative of oxotremorine (BM 123) to muscarinic receptors in the rat cerebral cortex. *Mol. Pharmacol.* **28**:107-119 (1985).
- Pedder, K. E., P. Eveleigh, D. Pouner, E. C. Hulme, and N. J. M. Birdsall. Modulation of the structure-binding relationships of antagonists for muscarinic acetylcholine receptor subtypes. *Br. J. Pharmacol.* **103**:1561-1567 (1991).
- Birdsall, N. J. M., S.-C. Chan, P. Eveleigh, E. C. Hulme, and K. W. Miller. The modes of binding of ligands to cardiac muscarinic receptors. *Trends Pharmacol. Sci.* (suppl.) **31**-34 (1989).
- Burgen, A. S. V. The role of ionic interactions at the muscarinic receptor. *Br. J. Pharmacol.* **25**:4-7 (1965).
- 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 analogues. *Mol. Pharmacol.* **2**:352-359 (1966).
- Ehler, F. J., and F. M. Delen. Influence of pH on the binding of scopolamine and N-methylscopolamine to muscarinic receptors in the corpus striatum and heart of rats. *Mol. Pharmacol.* **38**:143-147 (1990).
- Gupta, S., J. F. Moran, and D. J. Triggle. Mechanism of action of benzilylcholine mustard at the muscarinic receptor. *Mol. Pharmacol.* **12**:1019-1026 (1976).
- Barlow, R. B., and M. K. Shepherd. A further search for selective antagonists at M2-muscarinic receptors. *J. Pharm. Pharmacol.* **42**:412-418 (1986).
- Hammer, R., and A. Giachetti. Muscarinic receptor subtypes: M1 and M2 biochemical and functional characterization. *Life Sci.* **31**:2991-2998 (1982).
- Watson, M., H. I. Yamamura, and W. R. Roeske. A unique regulatory profile and regional distribution of [<sup>3</sup>H]pirenzepine binding in the rat provide evidence for distinct M1 and M2 muscarinic receptor subtypes. *Life Sci.* **32**:3001-3011 (1983).
- Giachetti, A., R. Micheletti, and E. Montagna. Cardiosensitive profile of AF-DX 116, a muscarinic M2-receptor antagonist. *Life Sci.* **38**:1663-1672 (1986).
- Giraldo, E., R. Micheletti, E. Montagna, A. Giachetti, M. A. Viganò, H. Ladinsky, and C. Melchiorre. Binding and functional characterization of the cardiosensitive muscarinic antagonist methoctramine. *J. Pharmacol. Exp. Ther.* **244**:1016-1020 (1988).
- Hammer, R., C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature (Lond.)* **283**:90-92 (1980).
- 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).

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