formation can be obtained with fluorescent alkylating agents (1). In addition, the *in vivo* activation mechanism for I*b* may account for the divergent physiological results of numerous other analogs (10).

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Interactions of Acetylcholine Mustard with Acetylcholinesterase

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Abstract □ The hydrolysis of acetylcholine and acetylcholine mustard by acetylcholinesterase was compared over a substrate concentration range of 1–10 m*M*. Reactions were allowed to proceed for 2 min at 25°. Results of these experiments reveal that the substrates have similar affinities for the enzyme, whereas the maximum velocity for the hydrolysis of acetylcholine mustard was significantly lower than for acetylcholine. These findings suggest that acetylcholine mustard has the ability to inactivate acetylcholinesterase.

Keyphrases □ Acetylcholine and acetylcholine mustard—hydrolysis by eel electroplax acetylcholinesterase □ Acetylcholinesterase—hydrolysis of acetylcholine and acetylcholine mustard

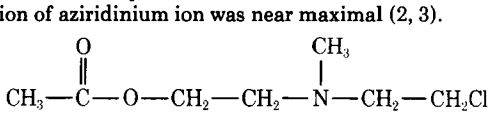
The synthesis of acetylcholine mustard [2-(chloroethylmethylamino)ethyl acetate] (I) was first reported by Hanby and Rydon (1). It was demonstrated that acetylcholine mustard can cyclize in buffered aqueous solutions to form an aziridinium ion (II) with alkylating ability (2, 3). This aziridinium ion is a close structural analog of acetylcholine.

In isolated muscle systems, it was found that acetylcholine mustard had about one-fifth the agonist potency of acetylcholine on the muscarinic receptors of the rat jejunum preparation or the nicotinic receptors of the frog rectus abdominis preparation (3). During a 1- or 2-hr exposure of the jejunum segments to either acetylcholine (1.0×10^{-4} *M*) or acetylcholine mustard (1.0×10^{-4} *M*), the contractions slowly declined; after washing, response to freshly applied acetylcholine was inhibited compared to control values. The decline in response was greater in the case of acetylcholine mustard, and the tissue did not fully recover even upon prolonged washing. Although the observed inhibition of response by acetylcholine and some of the effect of acetylcholine mustard could be accounted for by desensitization of receptors, the long-acting inhibition of the mustard was attributed to an irreversible inhibition brought about by alkylation of the receptors by the aziridinium ion (3).

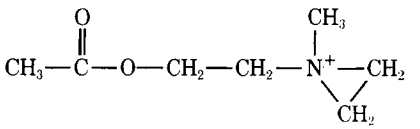
A comparable agonist effect of acetylcholine mustard on guinea pig ileum was found, but no irreversible inhibition was observed (2). An increase in the resting tonus of the muscle after exposure to the mustard was also observed and might have been due to an inhibition of acetylcholinesterase (2). Therefore, this study was undertaken to examine the interaction between acetylcholine mustard and acetylcholinesterase.

EXPERIMENTAL

In all experiments, acetylcholine mustard was dissolved in buffer solution and allowed to stand for 1 hr at room temperature before use. Previous experiments indicated that at this time the concentration of aziridinium ion was near maximal (2, 3).



I



II

Acetylcholinesterase Activity Assay—Acetylcholinesterase, prepared from electric eel[1], was used to catalyze the hydrolysis of acetylcholine or acetylcholine mustard. Disappearance of the substrate was determined by a colorimetric method (4). Briefly, to 2 ml of buffered substrate solution, 5 μl of acetylcholinesterase stock solution was added. The reaction was allowed to proceed for 2 min at 25° before it was stopped by the addition of freshly prepared alkaline hydroxylamine solution. After an additional 1 min, 4 *M* HCl and 0.37 *M* ferric chloride were added and the absorbance was read at 540 nm in a spectrophotometer.

Determination of Kinetic Constants—Estimations of kinetic constants for the hydrolysis of acetylcholine or acetylcholine mustard by acetylcholinesterase were made by determining the velocity of hydrolysis at substrate concentrations between 1 and 10 m*M*. At acetylcholine concentrations above 20 m*M*, substrate inhibition occurred. Kinetic constants were obtained by linear regression analysis of Hofstee plots of experimental data. The *F* test for "goodness of fit" for each curve was significant ($p < 0.05$).

Incubation of Enzymes with Substrates—In separate experiments, acetylcholinesterase was exposed to varying concentrations of either acetylcholine or acetylcholine mustard for 1 hr in buffer solution at 25° and then dialyzed against buffer at 0° for 2 hr to remove the remaining substrate or hydrolysis products. An aliquot of the enzyme solution was then taken for measurement of enzymatic activity as already described.

RESULTS AND DISCUSSION

Nickerson and Gump (5) were the first to demonstrate that the 2-haloethylamine moiety incorporated into α-adrenergic antagonists gave long-lasting activity. Ample evidence has been presented that this persistent antagonism is caused by formation of a covalent bond between the drug molecule in the form of a highly reactive aziridinium ion and a nucleophilic group on or near the receptor site (6–8).

Alkylation of the muscarinic receptor by an antagonist was first shown by Gill and Rang (9). These workers demonstrated that benzilylcholine mustard could produce selective and persistent antagonism of methylfurtrethonium-induced contractions of the isolated guinea pig ileum. Employing some elegant techniques, these workers found that the rate of dissociation of the aziridinium-ion–receptor complex was slow compared to the rate of receptor alkylation. Therefore, the mustard was a highly effective alkylating agent.

The acetylcholine analog, bromoacetylcholine, was shown to be capable of covalent bond formation with nicotinic receptors of eel electroplax (10). In this case, persistent stimulation rather than inhibition resulted. This example was apparently the first one of an irreversible agonist action at a receptor, although the agent was presumably covalently bound to a group just beyond the receptor site rather than within the receptor site proper. The aziridinium ion derived from acetylcholine mustard (II) is also a cholinergic agonist, but alkylation proceeded at a slow rate to yield a prolonged inhibition (3). It was decided to determine whether the acetylcholine mustard could also act as an irreversible inhibitor of acetylcholinesterase.

In some experiments, the effect of prolonged exposure of acetylcholinesterase to acetylcholine or acetylcholine mustard upon subsequent hydrolysis of acetylcholine was measured. In numerous trials, acetylcholinesterase was treated with either substrate as described under *Experimental*. The activity of the enzyme after such treatment proved to be considerably lower than that from controls using freshly prepared enzyme solutions. Although the hydrolysis rate was somewhat lower with enzyme exposed to the mustard than with enzyme exposed to acetylcholine, the difference was not significant.

In the next series of experiments, the initial hydrolysis rates for acetylcholine or acetylcholine mustard were determined at various concentrations of the two substrates. The Michaelis constant (K_m) and maximum velocity (V_m) were obtained by linear regression analysis of data from experiments in which the concentration of substrate was 1–10 m*M*. On the basis of these data (Table I), acetylcholine and the aziridinium ion from acetylcholine mustard appear to have similar affinities for acetylcholinesterase. This finding might be expected because of their close structural similarity. It is also evident from a comparison of the V_m's that acetylcholine mustard is hydrolyzed at a significantly lower maximum velocity than acetylcholine ($p < 0.001$).

Table I—Kinetic Constants for Hydrolysis of Acetylcholine and Acetylcholine Mustard, over a Substrate Concentration Range of 1—10 m*M*, Obtained by Linear Regression Analysis of Hofstee Plots of the Data

Substrate	K_m, m*M* ± *SE*	V_m, μmoles/min/mg ± *SE*	n[a]
Acetylcholine	1.05 ± 0.30	267.4 ± 19.2	166
Acetylcholine mustard	1.06 ± 0.44	135.8 ± 17.2	99

[a] Total number of observations at 1, 2, 3, 4, 5, 6, 8, and 10 m*M*.

In further experiments, the hydrolysis rate as a function of the substrate concentration was studied for acetylcholine, acetylcholine mustard, and a combination of equimolar parts of the two at a concentration range near the K_m. It can be seen in Fig. 1 that the relationship between velocity and substrate concentration is more nearly linear in the case of acetylcholine hydrolysis. A statistical comparison between the velocity of hydrolysis at each substrate concentration revealed acetylcholine was hydrolyzed significantly faster than acetylcholine mustard ($p < 0.001$).

The ratios of the velocities at two substrate concentrations (1.5 and 0.75 m*M*) for the hydrolysis of acetylcholine and acetylcholine mustard were 1.95 and 1.66, respectively. Thus, in the case of acetylcholine, the velocity of hydrolysis was nearly doubled as the substrate concentration was doubled; however, in the case of the mustard, there was a considerably smaller increase in velocity. The ratio of velocities at 1.5 and 0.75 m*M* substrate concentrations for the combination of acetylcholine and acetylcholine mustard was 1.88.

The question arises as to whether the lower V_m (Table I) and the curvature in the rate of hydrolysis at increasing acetylcholine mustard concentrations (Fig. 1) are caused by alkylation of the active site. It was shown (11) that acetylcholinesterase can be inhibited irreversibly by the alkylating agent *N,N*-dimethyl-2-phenylaziridinium chloride. This alkylating agent is not a close structural analog of acetylcholine, and alkylation may have taken place at an anionic site other than that at which the cationic head of acetylcholine normally binds to the enzyme. It is well known that certain quaternary ammonium ions can inhibit cholinesterase at sites different from the acetylcholine binding site (12). Alkylation of such allosteric sites might account for the irreversible inhibition by the aziridinium ion. In fact, O'Brien (13) summarized evidence

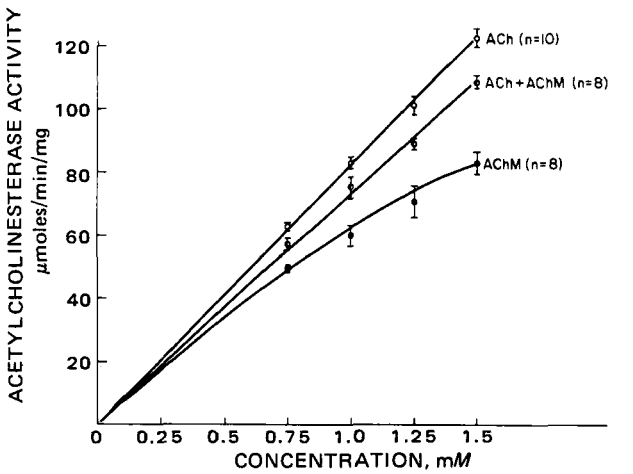


Figure 1—*Hydrolysis of acetylcholine (ACh), acetylcholine mustard (AChM), and an equimolar combination of acetylcholine and acetylcholine mustard (ACh + AChM) by acetylcholinesterase after 2 min of incubation at 25°. Numbers in parentheses refer to the number of separate experiments. Vertical bars represent standard error of the mean.*

[1] Type V, Sigma Chemical Co.

suggesting that the cationic head of acetylcholine may bind to the active site by nonpolar interactions rather than by ionic or polar forces. If such is the case, there may not be any nucleophilic group capable of being alkylated at this position on the enzyme.

Present results do not exclude the possibility that alkylation of the enzyme by the mustard could be occurring at sites other than the acetylcholine binding site. Alkylation by the mustard at the active site or at other sites would tend to lower the velocity of its hydrolysis. This was observed both in the experiments designed to provide kinetic constants (Table I) and in a separate series where each substrate and equimolar concentrations of each substrate were assayed (Fig. 1).

In summary, results of the present study reveal that acetylcholine is hydrolyzed at a significantly faster rate than the mustard, even though their affinities for the enzyme appear to be comparable. In the light of previous results obtained in cholinergic receptor systems, which demonstrated alkylation of a portion of receptors (3), it is possible that the mustard is capable of covalent bond formation with, and subsequent inactivation of, acetylcholinesterase. Due to rapid turnover of enzyme–substrate complexes, conditions for alkylation may not be favorable. In any case, it appears likely that alkylation and enzyme inactivation are occurring during hydrolysis of acetylcholine mustard by acetylcholinesterase. Hydrolysis of mixtures of the substrates would be expected to yield mixed-type inhibition.

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Effects of Nitrogen Dioxide and 3-Methylcholanthrene on Pulmonary Enzymes

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Abstract □ Guinea pig lung phenol-*O*-methyltransferase, catechol-*O*-methyltransferase, and benzpyrene hydroxylase activities were examined after nitrogen dioxide and 3-methylcholanthrene treatment. While benzpyrene hydroxylase activity was enhanced by 3-methylcholanthrene, none of the pulmonary enzyme activities was altered after exposure to either 40 or 70 ppm of nitrogen dioxide for 2 hr.

Keyphrases □ Nitrogen dioxide—effects on guinea pig lung phenol-*O*-methyltransferase, catechol-*O*-methyltransferases, and benzpyrene hydroxylase □ 3-Methylcholanthrene—effects on guinea pig lung enzymes □ Enzymes, pulmonary—effects of nitrogen dioxide and 3-methylcholanthrene

A previous report (1) from this laboratory demonstrated that guinea pig lung contains appreciable phenol-*O*-methyltransferase and catechol-*O*-methyltransferases. Furthermore, catechol-*O*-methyltransferases were present both in the supernatant and the microsomal fractions. The presence of these enzymes also has been indicated in rat lungs (2, 3). In addition, benzpyrene hydroxylase activity has been reported in rat lung (4) and is inducible by a wide range of chemicals including cigarette smoke (5), 3-methylcholanthrene (4, 6), flavones (7), and *Cannabis* (6).

The question was raised as to the influence of air pollutants on the primary pulmonary enzyme identi-

Table I—Pulmonary Enzyme Activities after 3-Methylcholanthrene Administration

Lung Enzymes[a]	Untreated Animals	3-Methylcholanthrene-Treated Animals
Benzpyrene hydroxylase	0.22 ± 0.19 (6)[b]	0.43 ± 0.26 (6)[c]
Microsomal phenol-*O*-methyltransferase	6.4 ± 0.72 (3)	7.2 ± 0.92 (3)
Supernatant catechol-*O*-methyltransferase	22.7 ± 1.4 (3)	22.9 ± 0.55 (3)
Microsomal catechol-*O*-methyltransferase	3.0 ± 0.17 (3)	3.6 ± 0.76 (3)

[a] Transmethylation activities are expressed as nanomoles of O-methylated substrates per gram of tissue; hydroxylation activities are expressed as fluorescence units per gram of tissue. [b] Values are given as means ± *SE*, with the number of animals used in parentheses. [c] Values are significantly different ($p < 0.1$) from untreated animals.