The Effect of Organic and Inorganic Cations on the Decarbamylation of Dimethylcarbamylacetylcholinesterase: A Comparison with Deacetylation

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SUMMARY

The effects of quaternary ammonium compounds and inorganic ions on the decarbamylation of dimethylcarbamylacetylcholinesterase at low ionic strength have been investigated. Both similarities and differences between the effects of these compounds on deacetylation and decarbamylation have been noted. Small quaternary ammonium compounds, such as tetraethylammonium and *cis*-2,6-dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide, accelerate decarbamylation, an effect which has also been observed with deacetylation. Larger quaternary ammonium compounds, such as tetrapropylammonium, have no effect on decarbamylation, whereas these compounds block deacetylation. Diethyldi(2-hydroxyethyl)ammonium iodide accelerates decarbamylation 71-fold, whereas this compound has a small inhibitory effect on deacetylation. NaCl and $MgCl_2$ accelerate decarbamylation, and organic and inorganic cations compete for the carbamylated enzyme. Different protein conformations of acetyl- and dimethylcarbamylacetylcholinesterase have been suggested.

INTRODUCTION

The experiments of Wilson (1) and Bergmann *et al.* (2) established that the hydrolysis of ester substrates proceeds through an acyl-enzyme intermediate. It is now generally accepted that organophosphates and at least some carbamates form corresponding phosphoryl- and carbamyl-enzyme intermediates (3, 4). In previous communications (5, 6) it was suggested that certain quaternary ammonium compounds potentiated the hydrolytic activity of acetylcholinesterase by accelerating the deacetylation step in the hydrolysis sequence. To obtain further evidence for this mechanism, the effect of these quaternary ammonium compounds on the decarbamylation of carbamylacetylcholinesterase has been studied, since it appeared probable that decarbamylation could serve as a model for deacetylation (7). The half-life of the carbamyl-enzyme is in the order of minutes (8, 9), whereas the half-life for the acetyl-enzyme is some micro-seconds (10). Thus decarbamylation is some 10^8 times slower than deacetylation, and the effect of cations on this reaction may be studied directly.

It has been shown (5, 6) that the presence of inorganic ions can mask the effects of quaternary ammonium compounds on acetylcholinesterase activity. In the present study the effect of quaternary ammonium compounds on decarbamylation has been examined in the absence of added inorganic ions. The effects of NaCl and $MgCl_2$ and mixtures of inorganic and organic cations on decarbamylation have also been investi-

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gated. A comparison of the effects of a number of compounds on deacetylation and decarbamylation has been made, and some similarities and differences between the effects of these compounds on the two reactions are reported here.

o-Nitrophenyl dimethylcarbamate was used to form dimethylcarbamylacetylcholinesterase. Bender and Stoops (11) reported that this substrate reacts stoichiometrically with acetylcholinesterase.

MATERIALS AND METHODS

o-Nitrophenyl dimethylcarbamate was prepared by the reaction of dimethylcarbamyl chloride (10.8 g) and *o*-nitrophenol (13.9 g) in dry pyridine (100 ml), according to Bender and Stoops (11). The material was recrystallized from petroleum ether; m.p. 57° [reported (11), 56.7–57.0°].

The following compounds were used without further purification: acetylcholine perchlorate, tetramethylammonium iodide, tetra-*n*-propylammonium iodide, and tetra-*n*-butylammonium iodide (British Drug Houses); and tetraethylammonium iodide (Hopkins and Williams). Phenyl acetate (British Drug Houses) was redistilled before use. Sodium chloride (British Drug Houses) and magnesium chloride (By-Products and Chemicals Proprietary, Ltd.) were of analytical reagent grade.

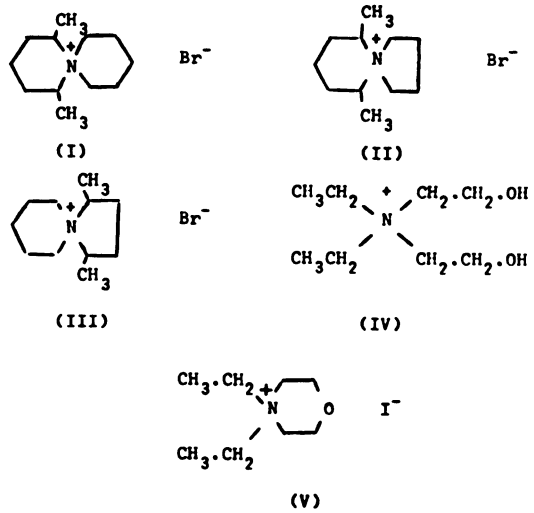
The syntheses of 2,6-dimethyl-1,1′-spirobipiperidinium bromide (compound I) and 2,6-dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide (II) have been described (5).

2,5-Dimethylspiro(pyrrolidine-1,1′-piperidinium)bromide (III) was prepared by the reaction of 2,5-dimethylpyrrolidine (2 mole equivalents) and 1,5-dibromopentane (1 mole equivalent) in redistilled methanol by autoclaving for 50 min at 125°. The total concentration of reactants was kept below 5% (w/v) to ensure intramolecular cyclization. The reaction solution was distilled to dryness under reduced pressure on a water bath, the solid residue was dissolved in water, sodium hydroxide (1 mole equivalent) was added, and the solution was distilled to dryness under reduced pressure on a water bath. The product was recrystallized from chloroform; m.p. 242–243°.

$C_{11}H_{22}BrN$
Calculated: C 53.2, H 8.9, N 5.6
Found: C 52.9, H 8.8, N 5.5

Diethyldi(2-hydroxyethyl)ammonium iodide (IV) was prepared by refluxing 2-diethylaminoethanol (1 mole equivalent) and 2-iodoethanol (2 mole equivalents) in dry methyl ethyl ketone for 6 hr. The solid which formed was filtered off and recrystallized from absolute ethanol; m.p. 230–231°.

$C_8H_{20}INO_2$
Calculated: C 33.2, H 7.0, N 4.8
Found: C 33.3, H 6.9, N 5.1



N,N-Diethylmorpholinium iodide (*V*) was prepared by refluxing morpholine (8.7 g) and ethyl iodide (16.0 g) in redistilled methanol (100 ml) for 6 hr. Sodium hydroxide (4 g) was added and stirred until dissolved. Ethyl iodide (16.0 g) was added, and the reaction mixture was refluxed for a further 6 hr. The resulting solid was recrystallized from absolute ethanol–ethyl methyl ketone; m.p. 246–247°.

$C_8H_{18}INO$
Calculated: C 35.4, H 6.7, N 5.2
Found: C 35.0, H 6.7, N 5.2

Enzymatic Procedure

o-Nitrophenyl dimethylcarbamate (0.04 ml, 3.25×10^{-4} M in 0.05% methanol) was added to 4.0 mg of bovine erythrocyte acetylcholinesterase (Nutritional Biochemicals Corporation). The mixture was incubated at 37° for 70 ± 10 min in a stoppered vessel. An aliquot (0.02 ml) was diluted to 40 ml with glass-distilled water or a solution containing the organic or inorganic compound under investigation. The pH was adjusted to 7.5 ± 0.1 and maintained at this value by the addition of 0.01 N NaOH. The solution was maintained at 36.3° under a stream of nitrogen. At suitable intervals over a period of up to 3 hr, aliquots of 3.2 ml were removed and diluted to 20 ml in a second jacketed vessel with a solution containing acetylcholine and sodium chloride. The concentrations of these in the final reaction mixture were 0.001, and 0.1 M, respectively. The remaining free acetylcholinesterase activity was then determined at pH 7.40 ± 0.05 and 37° titrimetrically, as previously described (12). A daily control was run, repeating the above procedure, but omitting the *o*-nitrophenyl dimethylcarbamate. A quaternary ammonium compound, used in the decarbamylation studies, was necessarily present during the determination of remaining free enzyme activity, but the inhibition of free enzyme activity caused by its presence was small, and appropriate corrections were made with similarly treated controls.

RESULTS AND DISCUSSION

The reaction between acetylcholinesterase and *o*-nitrophenyl dimethylcarbamate has been followed by Bender and Stoops (11). The kinetics of the release of *o*-nitrophenol was interpreted in terms of the following reaction sequence:

$$E + S \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} ES \xrightarrow{k'_2} ES' \xrightarrow{k'_3} E + P_2$$
$$\searrow P_1$$

where E is the free enzyme, S is the carbamate substrate, ES is the enzyme-substrate complex, ES' is the carbamyl-enzyme, P_1 is *o*-nitrophenol, and P_2 is *N*-dimethylcarbamic acid.

For the hydrolysis of *o*-nitrophenyl dimethylcarbamate, the ratio k'_2/k'_3 was found to be greater than 200. Decarbamylation is thus very much slower than carbamylation, and it is possible experimentally to study the decarbamylation reaction directly. Decarbamylation has been considered to be analogous to deacetylation (7), since in both cases an acyl-enzyme reacts with water to yield free enzyme and products. The two reactions are shown below:

$$E\text{—}COCH_3 + H_2O \xrightarrow{k_3} EH + CH_3COOH$$

$$E\text{—}CON(CH_3)_2 + H_2O \xrightarrow{k'_3} EH + CO_2 + (CH_3)_2NH$$

There is a large difference in the rates of the two enzymatic reactions (decarbamylation is 10^8 times slower than deacetylation), and both similarities and differences between the effects of added compounds on the two reactions might be expected.

Effect of tetraalkylammonium compounds. Figure 1 shows the effect of four tetraalkylammonium compounds on the rate of decarbamylation in the absence of added inorganic ions (ionic strength less than 0.005). The rates of decarbamylation were seen to follow pseudo first-order kinetics. Tetramethylammonium iodide, tetra-*n*-propylammonium iodide, and tetra-*n*-butylammonium iodide have no effect on the rate of decarbamylation. These compounds neither inhibit nor accelerate the

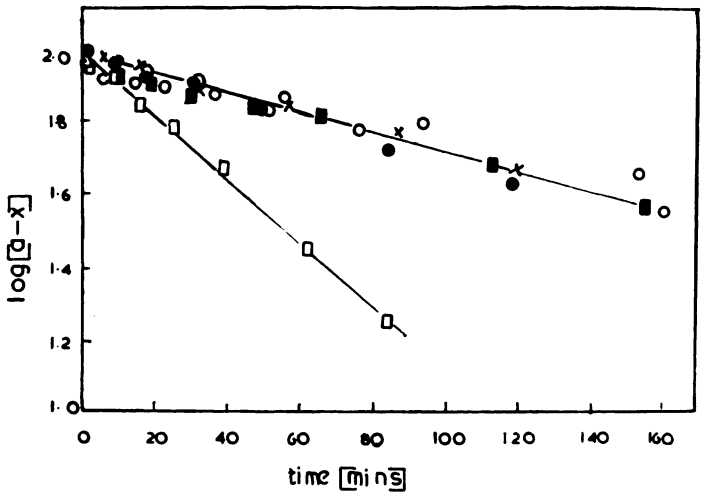


FIG. 1. *Effect of TMA, TEA, TPA, and TBA on decarbamylation of dimethylcarbamylacetylcholinesterase*

a is the percentage of carbamylated enzyme at $t = 0$, and x is the percentage of decarbamylation. The ionic strength of the control was less than 0.005. ○, Control; X, TMA, 5.60×10^{-3} M; ●, TPA, 1.11×10^{-4} M; ■, TBA, 4.44×10^{-5} M; □, TEA, 5.10×10^{-4} M.

decarbamylation reaction. On the other hand, in the presence of tetraethylammonium iodide, the rate of decarbamylation is accelerated about 4-fold. These results are given in Table 1, which compares the effect of the tetraalkylammonium compounds on deacetylation and decarbamylation. It can be seen that TMA[1] does not accelerate or inhibit either deacetylation or decarbamylation. TEA accelerates both deacetylation (2-fold) and decarbamylation (4-fold). The larger compounds TPA and TBA, however, have different effects on the two reactions; whereas TPA and TBA block deacetylation quite strongly, they have no effect on decarbamylation. Krupka (13) considered that large quaternary ammonium compounds, such as TPA, may block deacetylation by overlapping with the acetyl group on the enzyme and interfering with its reaction with water. If this explanation is correct, the failure of TPA and TBA to block decarbamylation may be due either to a situation in which the enzyme-carbamyl bond is at a distance from the anionic site greater than the length of a butyl chain (results obtained with compound IV make this unlikely) or to a situation in which the larger dimethylcarbamyl group sterically hinders overlapping of the propyl or butyl side chain with the acyl-enzyme bond. Probably a more satisfactory explanation is that groups larger than the ethyl groups of TEA cause unfavorable conformational perturbations of the acetyl-enzyme (12), thus inhibiting the rate of deacetylation. The protein structure of the carbamyl-enzyme may be already unfavorably perturbed by the dimethylcarbamyl group (compare rate difference of 10^8 in deacetylation and decarbamylation), and binding of even such large groups as TPA and TBA to the already perturbed enzyme may have little effect.

Effect of spiran quaternary ammonium compounds. In a previous communication (5) a high structural specificity was exhibited by a number of spiran quaternary ammonium compounds with respect to their ability to potentiate the hydrolysis of acetylcholine by acetylcholinesterase, and this specificity was related to their effect on the deacetylation reaction. Further evidence for

[1] The abbreviations used are: TMA, tetramethylammonium; TEA, tetraethylammonium; TPA, tetra-*n*-propylammonium; TBA, tetra-*n*-butylammonium.

TABLE 1
Effect of tetraalkylammonium compounds on decarbamylation of dimethylcarbamylacetylcholinesterase

ak'_3/k'_3 gives the ratio of the apparent first-order rate constant in the presence of the tetraalkylammonium compound to that in its absence. k'_3 represents the rate constant at ionic strength less than 0.005, and was found to be 5.2×10^{-3} min^{-1}. A value of 1.0 indicates no effect on decarbamylation; values greater than 1.0 indicate the degree of acceleration of decarbamylation.

Compound	Concentration	Effect on decarbamylation (ak'_3/k'_3)	Effect on deacetylation[a]
	M × 10^4		
TMA	11.2	1.3	None
	56.0	1.2	
TEA	5.1	3.9	2-Fold
	25.5	4.0	acceleration
TPA	0.111	1.2	Inhibition
	1.11	1.4	
TBA	0.444	1.1	Inhibition[b]

[a] Results from Roufogalis and Thomas (6).
[b] Result from Krupka (13).

this mechanism has since been obtained.[2] The effects of these compounds on decarbamylation have now been examined, and are compared with their effects on deacetylation in Table 2. These small spiran quaternary ammonium compounds exhibit identical specificities toward the two reactions. Thus compound I does not accelerate or inhibit either decarbamylation or deacetylation. Removal of one methylene group of the unsubstituted 6-membered ring of I to give a 5-membered ring gives II, which accelerates both decarbamylation (10-fold) and deacetylation (2-fold). Similarly, the transfer of the two methyl groups of II from the 6-membered ring to the 5-membered ring (III) results in loss of the ability of the molecule to accelerate decarbamylation, and the same effect was observed with deacetylation (Table 2). It thus appears likely that a similar mechanism is involved in the acceleration of both deacetylation and decarbamylation.

Effect of nucleophiles. A larger difference between acetyl- and dimethylcarbamylacetylcholinesterase was found when the effect of diethyldi(2-hydroxyethyl)ammonium iodide (IV) on the two reactions was studied. Compound IV blocks the deacetylation reaction weakly.[2] This compound, however, accelerates decarbamylation 71-fold (1.28×10^{-2} M). Cyclization of the two hydroxyl groups of IV to give V reduces potency in accelerating decarbamylation some 35-fold; the 2-fold acceleration of decarbamylation produced by this compound is in the same order as for the "non-nucleophilic" quaternary ammonium compounds, such as TEA. It thus appears likely that IV accelerates decarbamylation by a fundamentally different mechanism from the other quaternary ammonium compounds. One possibility is that the hydroxyl groups on IV accelerate decarbamylation via a nucleophilic attack on the carbamyl-enzyme bond, an interaction between the positive nitrogen of IV and the anionic site of the enzyme serving to orientate the hydroxyl group toward the carbamyl-enzyme bond. The ether oxygen in V is a much weaker nucleophile than the hydroxyl group of IV, but the conformational restriction in this compound could also be important to some extent in determining its lack of activity. The large acceleration of decarbamylation by IV is even more interesting in the light of the report of Wilson, Hatch, and Ginsburg (8) that pyridine-2-aldoxime methiodide, a potent reactivator of phosphorylated acetylcholinesterase, does not increase the rate of decarbamylation of dimethylcarbamylacetylcholinesterase. The hydroxylamine group of pyridine-2-aldoxime methiodide is more nucleophilic than the hydroxyl group of IV, but the inability of pyridine-2-aldoxime methiodide to accelerate decarbamylation may be related to an unfavorable orientation of the nucleophilic group of this molecule toward the carbamyl-enzyme bond. Unfavorable orientation of the nucleophilic group(s) of IV with the acetyl-enzyme bond may also explain the

[2] Unpublished observations.

TABLE 2

Effect of spiran quaternary ammonium compounds on decarbamylation of dimethylcarbamylacetylcholinesterase

ak'_3/k'_3 gives the ratio of the apparent first-order rate constant in the presence of the quaternary ammonium compound to that in its absence. k'_3 represents the rate constant at ionic strength less than 0.005, and was found to be 5.2×10^{-3} min^{-1}. A value of 1.0 indicates no effect on decarbamylation; values greater than 1.0 indicate the degree of acceleration of decarbamylation.

Compound	Concentration	Effect on decarbamylation (ak'_3/k'_3)	Effect on deacetylation[a]
	M × 10^4		
cis-2,6-Dimethyl-1,1'-spirobipiperidinium bromide (I)	1.1	1.3	None
	5.3	1.1	
cis-2,6-Dimethylspiro(piperidine-1,1'-pyrrolidinium) bromide (II)	2.5	5.5	2-Fold acceleration
	11.4	9.6	
2,5-Dimethylspiro(pyrrolidine-1,1'-piperidinium) bromide (III)	2.5	1.5	None
	12.5	0.96	

[a] Results from Roufogalis and Thomas (5) (and unpublished observations).

inability of this compound to accelerate the deacetylation of acetyl-acetylcholinesterase. If this is the case, it can be inferred that acetyl-, dimethylcarbamyl-, and phosphorylacetylcholinesterase may have different protein conformations, and that the spatial relationships of the anionic site and the acyl, carbamyl, and phosphoryl groups in all three intermediates may be different.

Effect of quaternary ammonium compounds on decarbamylation in the presence of inorganic ions. In the present investigation, in the absence of inorganic ions, TMA had no effect on the rate of decarbamylation. Wilson and Alexander (7), however, found that in a medium containing NaCl (0.1 M), $MgCl_2$ (0.02 M), gelatin (0.005%), and sodium phosphate (0.02 M), TMA inhibits decarbamylation quite strongly. In order to investigate the discrepancy between the results of Wilson and Alexander (7) and those obtained here, the effects of NaCl and $MgCl_2$ and mixtures of these ions and organic cations were investigated. The results are shown in Fig. 2 and Table 3. Figure 2 shows that NaCl and $MgCl_2$ accelerate the rate of decarbamylation 4- and 6-fold, respectively. This is the same order found for the acceleration of deacetylation by these ions, and the results serve as further evidence that inorganic ions potentiate acetylcholinesterase activity by accelerating the deacetylation step in the hydrolysis sequence (6, 14), since some similarity between deacetylation and decarbamylation has been shown. Sodium chloride (Table 3) and TEA (Table 1) both accelerate the rate of decarbamylation

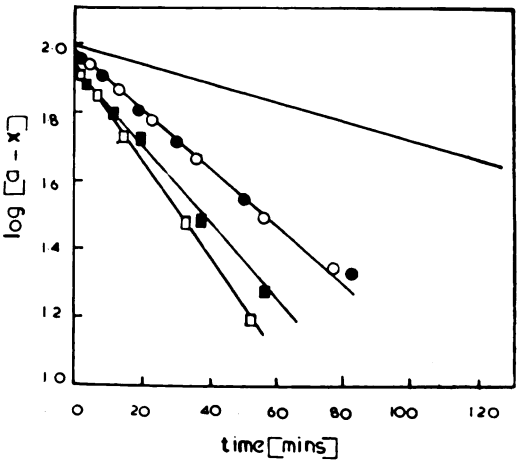


FIG. 2. *Effect of inorganic ions and mixtures of organic and inorganic ions on decarbamylation of dimethylcarbamylacetylcholinesterase*

The control curve is the same as in Fig. 1 (experimental points not shown). a is the percentage of carbamylated enzyme at $t = 0$, and x is the percentage of decarbamylation. ○, NaCl, 0.1 M; □, $MgCl_2$, 0.04 M; ●, NaCl, 0.1 M + TEA, 5.10×10^{-4} M; ■, $MgCl_2$, 0.04 M + TEA, 25.5×10^{-4} M.

TABLE 3

Effect of inorganic ions and mixtures of organic and inorganic cations on decarbamylation of dimethylcarbamylacetylcholinesterase

ak'_3/k'_3 gives the ratio of the apparent first-order rate constant in the presence of NaCl, $MgCl_2$, NaCl + TEA, and $MgCl_2$ + TEA to that in their absence. k'_3 represents the rate constant at ionic strength less than 0.005, and was found to be 5.2×10^{-3} min^{-1}. A value of 1.0 indicates no effect on decarbamylation; values greater than 1.0 indicate the degree of acceleration of decarbamylation.

Compound	Concentration	Effect on decarbamylation (ak'_3/k'_3)
	M	
NaCl	0.1	3.9
$MgCl_2$	0.04	6.0
NaCl + TEA	$0.1 + 5.10 \times 10^{-4}$	3.9
$MgCl_2$ + TEA	$0.04 + 25.5 \times 10^{-4}$	5.0

4-fold. If organic and inorganic cations compete for the same sites on acetylcholinesterase, possibly the free anionic site on the carbamyl-enzyme, the simultaneous addition of TEA and NaCl (at saturating concentrations) to the dimethylcarbamylacetylcholinesterase should produce acceleration of decarbamylation equal to their individual accelerations. This is what has been observed; a mixture of NaCl and TEA causes a 4-fold acceleration of decarbamylation (Fig. 2 and Table 3). $MgCl_2$ accelerates decarbamylation about 6-fold, whereas TEA accelerates it only 4-fold. If there is competition for a negative site on the carbamyl-enzyme, the simultaneous addition of both TEA and $MgCl_2$ to the dimethylcarbamylacetylcholinesterase should cause an acceleration intermediate between their individual accelerations. Again, this was the case, a mixture of $MgCl_2$ and TEA causing a 5-fold acceleration (Fig. 2 and Table 3). Thus, if the effect of TEA on decarbamylation was studied with respect to a control in the presence of $MgCl_2$, it would be concluded that TEA inhibits decarbamylation, whereas it has been shown in the present study that TEA accelerates decarbamylation. Similar effects on deacetylation were obtained with mixtures of organic and inorganic cations, and a similar scheme is now proposed:

$$\begin{array}{ccccc} EIS' & \rightleftharpoons & ES' & \rightleftharpoons & EMS' \\ \downarrow ak'_3 & & \downarrow k'_3 & & \downarrow bk'_3 \\ E + I + P & & E + P & & E + M + P \end{array}$$

where *ES'* is the carbamylated enzyme, *EMS'* is the carbamyl-enzyme-metal ion complex,[3] and *EIS'* is the carbamyl-enzyme-organic cation complex. *EIS'* and *EMS'* are decarbamylated, respectively, at a and b times the rate of decarbamylation of *ES'*. The inhibition of decarbamylation by TMA found by Wilson and Alexander (7) in the presence of inorganic ions can be explained by this scheme, since TMA, which does not itself accelerate decarbamylation, will compete with Na^+ and Mg^{++} for the carbamyl-enzyme and so reduce their accelerating effect.

In summary, the effects of cationic compounds on the rates of decarbamylation and deacetylation exhibited both similarities and differences, depending upon the structure of the cation. Thus tetraalkylammonium compounds of the size of TEA or smaller, spiran quaternary ammonium compounds with 5- or 6-membered rings, and inorganic ions have similar effects on the two reactions. Inorganic ions (NaCl and $MgCl_2$) and some of the quaternary ammonium compounds accelerate the rate of decarbamylation. A high degree of structural specificity is exhibited among the spiran quaternary ammonium compounds with respect to their ability to accelerate decarbamylation, as was found with deacetylation. Larger quaternary ammonium compounds, such as TPA and TBA, and quaternary ammonium compounds having nucleophilic groups show considerable dif-

[3] The formation of complex *EMS'* is inferred from evidence that metal ions reduce the affinity of acetylcholinesterase for acetylcholine (15, 16) and for cationic inhibitors (17, 18), presumably because of competition between organic and inorganic cations for the anionic site on the free enzyme. By analogy, it is likely that organic and inorganic cations will also compete for the free anionic site on the carbamylated enzyme.

ferences in their effects on the two reactions. Organic and inorganic cations compete for the dimethylcarbamylacetylcholinesterase, and the presence of inorganic ions can mask the effect of the quaternary ammonium compounds being studied.

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