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3 [ THE REACTIVATION OF DIMETHYLCARBAMOYL - ACETYLCHOLINESTERASE ] [R]

by

4 [ B.C. Barrass, <sup>et al</sup> B.R. Cole and D.B. Coult

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DATE: OCTOBER 1971

THE REACTIVATION OF DIMETHYLCARBAMOYL-ACETYLCHOLINESTERASE

by

B. C. BARRASS, B. R. COLE AND D. B. COULT

SUMMARY

Several quaternary ammonium salts have been prepared as possible enzyme reactivators for the treatment of carbamate poisoning. Compounds which possess one or more free hydroxy groups accelerated the rate of spontaneous reactivation, but compounds lacking hydroxy groups either had no effect or actually inhibited the rate of spontaneous reactivation. It is concluded that the acceleration factors observed were sufficiently high to warrant testing of the compounds in vivo.

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INTRODUCTION

Very highly toxic carbamates have been known for many years and although it is considered that the physical properties of those substances which have been prepared so far militate against their use in large scale chemical warfare, nevertheless the existence of such a class of compound poses problems which cannot be ignored. The difficulty of treating carbamate poisoning is one of these problems and the present paper is concerned primarily with this aspect. An additional interest in carbamates has arisen from the suggestion that they may be of use (1) prophylactically for minimising or counteracting the toxic effects of nerve agents. It is interesting therefore to look also at the possibility that drugs having a specific effect against carbamate poisoning could also be used to modify the duration of action of carbamates employed for the purpose of nerve agent prophylaxis.

It is now well established (2) that the effects of carbamates on acetylcholinesterase (EC 3117) involve the formation of a carbamoylated enzyme (I)



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Clearly, any compound which would accelerate the rate of spontaneous reactivation of I might be expected to exert a beneficial effect in cases of carbamate intoxication; such an expectation is reinforced by the success which has attended the development of specific therapeutic drugs (oximes) for the biochemically analogous case of intoxication by some organophosphates (3). Previous work (4,5,6) has shown that some quaternary ammonium salts, particularly those which contain hydroxy groups, accelerate the deacetylation and decarbamylation of acetylated and carbamoylated acetylcholinesterase. These studies were, however, carried out in solutions of very low ionic strength and it is possible that the effects observed were due solely to the effects of the quaternary salts on the ionic strength of the medium (7). It was therefore decided to repeat this work under more realistic conditions in solutions of an ionic strength approximating to that which obtains under physiological conditions. In this paper an account is given of these investigations, and the significance of the results obtained is discussed.

## EXPERIMENTAL

### Materials

The enzyme used was purified bovine erythrocyte acetylcholinesterase (EC 3117) obtained from Sigma Chemical Company. Neostigmine methyl sulphate was obtained from Koch-Light Ltd. The remaining compounds used were prepared at CDE by published procedures (4,8); the identities of the compounds were confirmed by melting points and by spectroscopic examination.

### Methods

O-Nitrophenyl N,N-dimethylcarbamate (8) was dissolved in anhydrous methanol to give a  $2 \times 10^{-3}$  M stock solution. An

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aliquot (0.03 ml) of this stock solution was added to a 0.1M aqueous solution of sodium chloride (9.97 ml) containing 10 $\mu$ M units of acetylcholinesterase (3.6 mg of the Sigma preparation) at pH 7.4 and 37 $^{\circ}$ . At appropriate time intervals aliquots (0.5 ml) of this incubation mixture were added to 0.9% aqueous sodium chloride (14.5 ml) containing sufficient acetyl choline iodide to give a final substrate concentration of  $6 \times 10^{-4}$  M. This assay solution was contained in a Radiometer pH-stat cell and the assay was carried out at pH 7.4 and 37 $^{\circ}$ . The pH-stat was equipped with a twin-syringe drive, the syringes containing  $10^{-2}$  M aqueous acetyl choline iodide and  $10^{-2}$  M aqueous sodium hydroxide respectively. These two solutions were thus added to the assay mixture at identical rates, ensuring that the assay was carried out at constant substrate concentration with a consequent improvement in accuracy and reproducibility (9).

These experiments were repeated in the absence of the carbamate, with the various quaternary ammonium salts (modifiers) replacing the carbamate in the incubation solution and also with both the carbamate and the modifier present simultaneously in the incubation solution. In all cases the volumes were adjusted so that the incubation solution had an initial total volume of 10 ml and the assay solution a volume of 15 ml; the aliquots taken from the incubation solution for assay were always 0.5 ml.

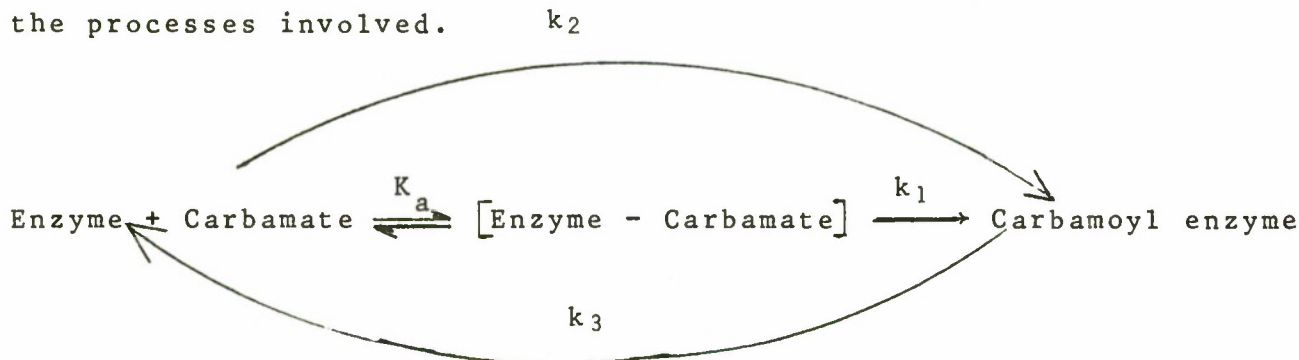
Similar procedures were employed when neostigmine was used as the inhibitor. In this case the inhibitor concentration was  $10^{-5}$  M and the incubation mixture was 0.154M in sodium chloride.

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RESULTS

The effects on the rates of carbamylation and decarbamylation of acetylcholinesterase, using  $10^{-3}$  M concentrations of the various quaternary ammonium salts (enzyme modifiers) listed in Table 1, are summarised in Tables 1 and 2. The rates given were calculated on the assumption (10) that the following reaction scheme is an adequate representation of the processes involved.



In the presence of excess carbamate  $k_2$ , which is a complex constant containing both  $K_a$  and  $k_1$ , may be considered as the rate coefficient of a first order reaction which is opposed by another first order reaction with a rate coefficient  $k_3$ . Under these conditions  $k_2$  and  $k_3$  may be obtained from the following equations (11)

$$k_{\text{obs}} = k_2 + k_3 = 1/t \ln x_0/x_e - x$$

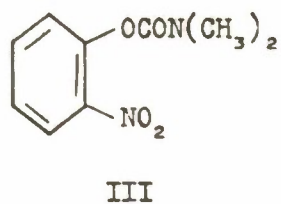
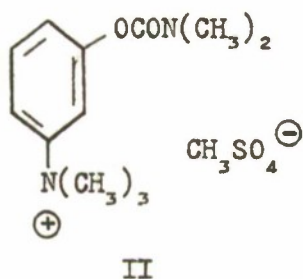
$$\text{and } k_2/k_3 = x_0/100 - x_e$$

where  $x_0$  = equilibrium percentage of original enzyme activity and  $x$  = percentage of original enzyme activity at time  $t$ .  $K_a k_1$  was obtained by dividing the value for  $k_2$  by the carbamate concentration.

The values of  $k_3$  obtained using neostigmine (II) and O-nitrophenyl N,N-dimethylcarbamate (III), shown in Tables 1 and 2 respectively, were in good agreement as also were the

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values for the  $k_3$  acceleration factors. This was expected since both inhibitors should give the same dimethylcarbamoyl enzyme.



The acceleration factors given in Tables 1 and 2 were obtained by dividing the value for  $k_3$  obtained in the presence of the enzyme modifier by the value for  $k_3$  derived from the unmodified reaction.

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TABLE I

RATE COEFFICIENTS AND RATE CONSTANTS USING NEOSTIGMINE AS INHIBITOR

Modifier	$k_{obs}$ ( $\text{min}^{-1}$ )	$k_2$ ( $\text{min}^{-1}$ )	$K_a k_1$ ( $\text{l.mol}^{-1}\text{min}^{-1}$ )	$k_3$ ( $\text{min}^{-1}$ )	Acceleration factor
None	0.108	0.081	$3.6 \times 10^6$	0.027	-
$(\text{C}_2\text{H}_5)_4\text{N}^+\cdot\text{I}^-$	0.071	0.044	$1.5 \times 10^6$	0.027	1.0
$(\text{C}_2\text{H}_5)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH}\cdot\text{I}^-$	0.0895	0.051	$1.7 \times 10^6$	0.0385	1.4
$(\text{C}_2\text{H}_5)_2\text{N}^+(\text{CH}_2\text{CH}_2\text{OH})_2\cdot\text{I}^-$	0.124	0.056	$1.9 \times 10^6$	0.068	2.5
$(\text{C}_2\text{H}_5)_2\text{N}^+(\text{C}_6\text{H}_{11}\text{O})\cdot\text{I}^-$	0.0757	0.054	$1.8 \times 10^6$	0.0217	0.8
$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH}\cdot\text{I}^-$	0.134	0.0453	$1.5 \times 10^6$	0.089	3.3
$(\text{CH}_3)_2\text{N}^+(\text{CH}_2\text{CH}_2\text{OH})_2\cdot\text{I}^-$	0.161	0.0462	$1.54 \times 10^6$	0.115	4.3
$(\text{CH}_3)_2\text{N}^+(\text{C}_6\text{H}_{11}\text{O})\cdot\text{I}^-$	0.0723	0.0516	$1.72 \times 10^6$	0.0208	0.77

Neostigmine concentration =  $3 \times 10^{-8}$  M, pH 7.4, 37°

Sodium chloride = 0.154M

Modifier concentration =  $10^{-3}$  M

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TABLE 2

RATE COEFFICIENTS AND RATE CONSTANTS USING O-NITROPHENYL  
N,N-DIMETHYLCARBAMATE AS INHIBITOR

Modifier	$k_{obs}$ ( $\text{min}^{-1}$ )	$k_2$ ( $\text{min}^{-1}$ )	$K_a k_1$ ( $1.\text{mol}^{-1}\text{min}^{-1}$ )	$k_3$ ( $\text{min}^{-1}$ )	Acceler- ation factor
None	0.135	0.110	$1.7 \times 10^4$	0.0252	-
$(\text{C}_2\text{H}_5)_4\text{N}^{\oplus} \text{I}^{\ominus}$	0.100	0.0714	$1.1 \times 10^4$	0.0287	-
$(\text{C}_2\text{H}_5)_3\text{N}^{\oplus}\text{CH}_2\text{CH}_2\text{OH} \text{I}^{\ominus}$	0.111	0.0712	$1.1 \times 10^4$	0.0403	1.6
$(\text{C}_2\text{H}_5)_2\text{N}^{\oplus}(\text{CH}_2\text{CH}_2\text{OH})_2 \text{I}^{\ominus}$	0.153	0.0818	$1.3 \times 10^4$	0.0712	2.8
$(\text{CH}_3)_3\text{N}^{\oplus}\text{CH}_2\text{CH}_2\text{OH} \text{I}^{\ominus}$	0.150	0.0645	$1.0 \times 10^4$	0.0855	3.4

O-Nitrophenyl N,N-dimethylcarbamate concentration =  $6.48 \times 10^{-6}$  M, pH 7.4, 37°.

Sodium chloride = 0.1M.

Modifier concentration =  $10^{-3}$  M.

In the experiments with no carbamate present the modifiers produced a very small degree of inhibition which remained at a constant level throughout the time period of the measurements.

A plot of  $1/i$  ( $i$  = inhibitor concentration) v.  $t/2.303 \Delta \log v$  ( $\Delta \log v$  = the difference between the logarithms of the velocities of the enzymic reaction after 3 minutes in the presence and absence of inhibitor (12)) was linear and was used to evaluate  $1/k_1$  and  $1/K_a$  using the equation shown below (13), where  $K_a$  and  $k_1$  have the significance shown in the previous scheme.

$$1/i = t/2.303 \Delta \log v \cdot k_1/K_a - 1/K_a$$

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The values obtained for acetylcholinesterase using O-nitrophenyl N,N-dimethylcarbamate as inhibitor were  $K_a = 5 \times 10^{-5}$  M,  $k_1 = 0.9 \text{ min}^{-1}$ .

## DISCUSSION

The results listed in Tables 1 and 2 show that certain quaternary ammonium salts will catalyse the spontaneous reactivation (decarbamoylation) of acetylcholinesterase which has been inhibited by neostigmine or O-nitrophenyl N,N-dimethylcarbamate. This effect is only observed with those compounds which possess a free hydroxy group; compounds which do not possess one or more hydroxy groups, or where these groups are combined in an ether linkage, either have no effect on spontaneous reactivation or actually inhibit it. The fact that the hydroxy groups are so important suggests that they play an active role in promoting the decarbamoylation of carbamoyl acetylcholinesterase and three possibilities for the mechanism of action of these quaternary compounds may be envisaged.

It is possible that the hydroxy groups are acting as nucleophiles and carry out a nucleophilic displacement reaction on the carbamoyl group in much the same way as oximes displace the organophosphorus moiety from phosphorylated acetylcholinesterase (3). This is perhaps unlikely in view of the probably high  $pK_a$  of the hydroxy groups, at least in the bulk solution; what the value of the  $pK_a$  of these groups is when the molecule is complexed with the enzyme is conjecture only at this stage.

An alternative, and more likely, possibility is that the hydroxy groups are assisting nucleophilic attack by a water molecule on the carbamoyl carbonyl group either by hydrogen

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bonding assistance or by locally modifying the environment around the carbonyl group. In this context it would be of interest to repeat the experiments using a monomethyl rather than a dimethyl carbamate. It has been suggested that the hydrolysis of monomethyl (and unsubstituted) carbamates proceeds initially by abstraction of a proton from the NH group of the carbamate followed by fission of the labile C - O bond (14) and it is possible that a similar mechanism could be effective in causing the spontaneous reactivation of acetylcholinesterase inhibited by monomethyl carbamates.

If the quaternary ammonium salts studied in this investigation are acting as nucleophiles then it might be anticipated that the acceleration factors observed with monomethyl and dimethyl carbamates may not be too dissimilar. If, on the other hand, the quaternary salts are acting by a mechanism involving hydrogen bonding or an alteration in the environment around the carbamoyl group attached to the enzyme then the acceleration factors may be greater with monomethyl than with dimethyl carbamates. It should be pointed out that the acceleration factors obtained in the present work, which was carried out at a relatively high ionic strength, are much smaller than those reported by other workers (2) who used much lower ionic strengths. This initial dependence upon ionic strength suggests that a mechanism other than nucleophilic displacement is operative. It would be of interest to repeat these studies over a range of ionic strengths to discover precisely how the acceleration factor varied; such studies would assist in the elucidation of the mechanism by which the quaternary salts catalyze spontaneous reactivation.

A final possibility, on which there is at present little direct evidence, is that the acceleration may be wholly allosteric in nature (5,15). It is known that acetylcholinesterase exists in various forms, the nature and relative proportions of which depend on the ionic strength of the

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enzyme solution (6); it would be of considerable interest to extend such studies on allosteric forms to include the quaternary ammonium salts described in this report.

The rate of uncatalysed spontaneous reactivation ( $k_3$ ) of N,N-dimethylcarbamoyl acetylcholinesterase is in good agreement with that found by previous workers (10,12). Since the plot of  $1/i \ t/2.303 \ \Delta \log v$  was linear in the case of inhibition by O-nitrophenyl N,N-dimethylcarbamate it is likely that in this case as well as in the case of neostigmine (13) the reversible formation of an enzyme-inhibitor complex precedes the formation of the carbamoylated enzyme.

It has already been mentioned that the acceleration factors observed in the present investigations are smaller than those observed by other workers(4). Nevertheless the fact that acceleration factors of up to 4 were obtained under the more realistic conditions of the present investigation suggests that some of these quaternary ammonium salts may be of therapeutic use in the treatment of carbamate intoxication. The necessary animal testing is being carried out and will be reported separately.

## CONCLUSIONS

1. Quaternary ammonium compounds which contain one or more free hydroxy groups accelerate the spontaneous reactivation of N,N-dimethylcarbamoyl acetylcholinesterase, but the mechanism by which they produce this effect is not known.
2. Although the acceleration factors observed were smaller than those previously reported by other workers they are sufficiently high to warrant testing of these compounds for their therapeutic efficacy in the treatment of carbamate poisoning.

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