

## INFLUENCE OF TEMPERATURE AND CHOLINERGIC LIGANDS ON THE CARBAMYLATION OF UNMODIFIED AND CARBODIIMIDE-MODIFIED ERYTHROCYTE ACETYLCHOLINESTERASE

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### 1. Introduction

A number of previous studies have shown that the hydrolysis of acetylcholine by acetylcholinesterase (EC 3.1.1.7) from a number of sources is accelerated by selected ligands [1–3]. It was suggested by one of us that this acceleration occurs at the deacetylation step in the reaction sequence [2,4–6], by binding of the ligands at an allosteric site [7]. Recently we have shown that this site could be selectively blocked by a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) [8]. It was possible, however, that the ligands may also accelerate the rate of acetylation of acetylcholinesterase by substrates [9].

In this communication, we have shown that only the neuromuscular blocking agents accelerate carbamylation of the esteratic site of the enzyme by a neutral carbamate, whereas inorganic ions have no effect and small quaternary ammonium compounds inhibit this reaction. All of the ligands examined inhibited carbamylation by neostigmine. We have demonstrated that the same allosteric site that functions in the acceleration of deacetylation of the enzyme also functions in acceleration of carbamylation, a reaction considered analogous to acetylation [10]. The influence of temperature on the ligand-induced activation and inhibition of the carbamylation was studied in the hope of gaining further information on the related mechanisms occurring at the cholinergic receptor [11].

### 2. Materials and methods

#### 2.1. Source of enzyme

Acetylcholinesterase was a partially purified bovine erythrocyte preparation from Sigma. At low ionic strength, it had a specific activity of 156  $\mu$ moles of acetylcholine hydrolyzed per hr per mg of protein and a  $K_M$  of 22  $\mu$ M. The EDAC-modified enzyme was prepared as previously described [8] in a concentration of 1 mg/ml, except that the reaction time was increased to 7.5 hr to ensure near maximum modification.

#### 2.2. Source of chemicals

*o*-Nitrophenoldimethylcarbamate (NPDC) was prepared as previously described [6]. Inorganic ions were of analytical reagent grade. Tetramethylammonium (TMA) and tetraethylammonium (TEA) iodide (Baker), decamethonium bromide (K & K) and neostigmine bromide (Sigma) were used as received. Gallamine triethiodide was a gift from Poulenc Ltd. Acetyl-[1- $^{14}$ C]choline iodide (New England Nuclear) had a specific activity of 4.53 Ci/mole. Cation exchange resin CGC 241, Na<sup>+</sup> form, 200–400 mesh was Reagent Grade from Baker.

#### 2.3. Carbamylation measurements

Carbamylation was measured essentially by reaction of acetylcholinesterase with the carbamate in the jacketed microvessel of the Radiometer TTA 31 in the absence of added ions at pH 7.4, and 25°C, with removal of aliquots for radiometric assay at required time periods. Specifically, 3 mg of enzyme preparation in 3.0 ml of glass distilled water was adjusted to pH 7.4 in the microvessel. Two 10  $\mu$ l aliquots were removed

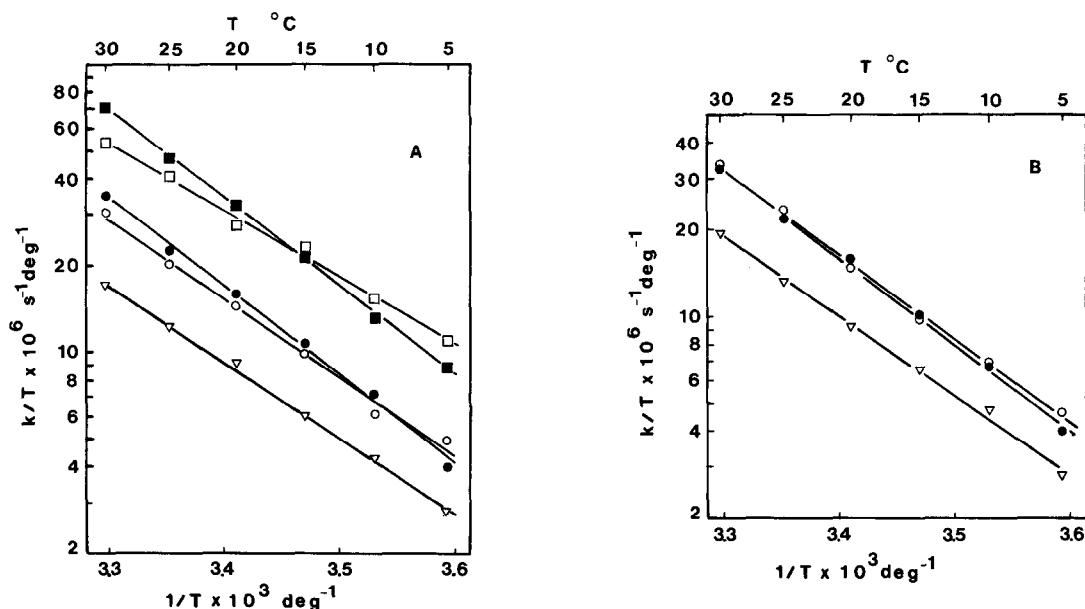


Fig. 1. Effect of temperature on the apparent carbamylation rate constant. A) Control,  $\circ$ ; 0.5 mM TMA,  $\Delta$ ; 50 mM NaCl,  $\bullet$ ; 0.1  $\mu\text{M}$  decamethonium,  $\square$ ; 10  $\mu\text{M}$  gallamine,  $\blacksquare$ . B) 40 mM  $\text{MgCl}_2$ ,  $\circ$ ; 0.2 mM  $\text{CaCl}_2$ ,  $\bullet$ ; 0.5 mM TEA,  $\Delta$ .

and assayed for control activity as described below. At zero time 30  $\mu\text{l}$  of an 8.1 mM solution of *o*-nitrophenoldimethylcarbamate in 5% ethanol or a 7.5  $\mu\text{M}$  solution of neostigmine was added to the reaction vessel (final concentrations  $8.1 \times 10^{-5} \text{ M}$  and  $7.5 \times 10^{-8} \text{ M}$ , respectively). At suitable time intervals (20–90 sec), 10  $\mu\text{l}$  aliquots were removed and assayed for remaining enzyme activity. The time period of reaction varied from 4–10 min.

### 2.3.1. Radiometric assay

The 10  $\mu\text{l}$  aliquots were added to tubes containing 0.22 ml of 0.1 M sodium phosphate buffer, pH 7.4 and a mixture of acetylcholine perchlorate and acetyl-[1- $^{14}\text{C}$ ]choline iodide to a final total substrate concentration of 1 mM, containing 19.0 nCi per tube. After 2 min at room temperature the reaction was stopped by the addition of 0.2 ml absolute ethanol. Tubes were then assayed for remaining free enzyme activity by a modification of the procedure of Berg and Maickel [12], as previously described [13].

## 3. Results

Fig. 1 shows Arrhenius-type plots for the carbamylation of erythrocyte acetylcholinesterase by NPDC for the control reaction and in the presence of 0.05 M NaCl, 0.5 mM TMA, 0.1  $\mu\text{M}$  decamethonium and 10  $\mu\text{M}$  gallamine (fig. 1A) and 0.04 M  $\text{MgCl}_2$ , 0.2 mM  $\text{CaCl}_2$  and 0.5 mM TEA (fig. 1B). In all cases, the plots have been drawn by regression analysis and correlation coefficients were found to be better than 0.99. None of the inorganic ions had any effect on the NPDC carbamylation rates at the temperatures studied (5–30 $^{\circ}\text{C}$ ). The small quaternary ammonium compounds, TMA and TEA, inhibit carbamylation rates at all temperatures. The neuromuscular blocking drugs, decamethonium and gallamine, accelerate carbamylation rates about two-fold. Inorganic ions, TMA and TEA, and the neuromuscular drugs inhibited the carbamylation of acetylcholinesterase by neostigmine, presumably by interfering with the binding of the quaternary ammonium head of this inhibitor (table 1).

Table 1 also shows the effect of the ligands on the carbamylation (by NPDC) of the EDAC-modified enzyme. Whereas the inhibition of this reaction by TMA and TEA is essentially unaffected, acceleration of the

Table 1

Comparison of effects of ligands on carbamylation of acetylcholinesterase by *o*-nitrophenoldimethylcarbamate and neostigmine.

Ligand (mM)	Effect on carbamylation rate <sup>a</sup>		
	NPDC		Neostigmine
	Unmodified	EDAC- modified <sup>b</sup>	Unmodified <sup>c</sup>
TMA (0.5)	0.64	0.78	0.24
TEA (0.5)	0.64	0.73	0.21
CaCl <sub>2</sub> (0.2)	1.1	—	0.52
MgCl <sub>2</sub> (40)	1.0	—	0.11
NaCl (50)	1.1	—	0.26
Gallamine (0.01)	2.3	1.6	0.27
Decamethonium (0.0001)	1.9	1.3	0.49

<sup>a</sup> A value of 1.0 represents no effect, less than 1.0 represents degree of inhibition and greater than 1.0 the degree of acceleration. Results refer to reactions at 20°C.

<sup>b</sup> Carbamylation rate of the EDAC-modified enzyme at 20°C is  $4.2 \times 10^{-3} \cdot \text{sec}^{-1}$ , compared to  $7.5 \times 10^{-3} \cdot \text{sec}^{-1}$  for the control.

<sup>c</sup> The carbamylation rate is  $6.2 \times 10^{-3} \cdot \text{sec}^{-1}$ .

reaction of the unmodified enzyme by gallamine and decamethonium is greatly diminished after EDAC-modification. The small amount of acceleration still remaining is probably due to incomplete modification of the enzyme by EDAC even after 7.5 hr of reaction.

Table 2 shows the thermodynamic parameters  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  for the NPDC carbamylation in the presence of the various ligands, determined from the slopes and intercepts, respectively, of the Arrhenius-type plots in fig. 1A and B, as previously described [13].

#### 4. Discussion

The present data confirm our previous suggestion [2,4,5] that the influence of ligands on substrate hydrolysis is determined by the rate limiting step in the hydrolysis sequence of that substrate and the conditions (such as substrate concentration and ionic strength) under which the reaction is investigated. Thus inorganic ions (CaCl<sub>2</sub>, MgCl<sub>2</sub> and NaCl) which accelerate the decarbamylation of acetylcholinesterase [6] have no effect on the carbamylation of the enzyme

Table 2

Effect of ligands on carbamylation rates and thermodynamic parameters for acetylcholinesterase inhibition.

Ligand (mM)	Relative carb- amylation rate <sup>a</sup>	$\Delta H^\ddagger$ kcal/mole	$\Delta S^\ddagger$ e.u.
Control	1.00	12.8	-25.6
TMA (0.5)	0.64	12.0	-29.3
TEA (0.5)	0.64	12.4	-28.0
CaCl <sub>2</sub> (0.2)	1.1	13.8	-22.2
MgCl <sub>2</sub> (40)	1.0	13.4	-23.7
NaCl (50)	1.1	13.6	-23.0
Gallamine (0.01)	2.3	14.1	-19.9
Decamethonium (0.0001)	1.9	10.6	-31.9

<sup>a</sup> As in table 1. Results represent carbamylation by NPDC at 20°C.

by the uncharged carbamate (table 1) but inhibit to various degrees the reaction by neostigmine (either by direct competition for binding of the quaternary ammonium head at the catalytic anionic site or by anticooperative interaction between anionic sites) (table 1). As would be predicted from these data on the partial reactions of acetylcholinesterase, inorganic ions accelerate acetylcholine hydrolysis at high substrate concentrations [5,9], where deacetylation is considered rate limiting [14], but inhibit acetylcholine hydrolysis at low substrate concentrations (where deacetylation is not rate limiting [15]). NaCl also inhibits hydrolysis of a poor quaternary ammonium substrate where deacetylation cannot be rate limiting [4], presumably by interfering with the binding of the quaternary ammonium group to the enzyme. Similarly, TEA, which in bovine erythrocyte acetylcholinesterase accelerates decarbamylation\* (at low ionic strength), but inhibits carbamylation by both the neutral and positively charged carbamates\* (table 1), accelerates acetylcholine [5,10] and phenylacetate [2] hydrolysis at high substrate concentrations, but inhibits their

\* It seems that TEA does not accelerate either decarbamylation [3] or acetylcholine hydrolysis [9] in electric eel acetylcholinesterase, probably reflecting a species difference between these enzymes [9]. In addition, TEA has been found to accelerate the reaction of electric eel acetylcholinesterase by methanesulfonyl fluoride [11] and dimethylcarbamyl fluoride [3,10], an effect that may be related to the presence of the small fluoride leaving group of these inhibitors.

hydrolysis at low substrate concentrations [2,5]. TEA also inhibits the hydrolysis of a poor quaternary ammonium substrate at all substrate concentrations [5]. The situation may be somewhat more complex with the neuromuscular blocking agents. Thus, whereas decamethonium inhibits decarbamylation [3] and the reaction of the enzyme with neostigmine (table 1), it accelerates carbamylation by the neutral carbamate. Decamethonium is a mixed (competitive and noncompetitive) inhibitor of acetylcholine hydrolysis [7], a result which is consistent with its effects on the corresponding partial reactions discussed above. It would however be of interest to study the effect of decamethonium on neutral substrates at low substrate concentrations in case some acceleration is found. Gallamine, which is one of the more potent accelerators of decarbamylation [3,13] and carbamylation by NPDC (table 1), but inhibits reaction of the enzyme with neostigmine (table 1), accelerates acetylcholine hydrolysis at high substrate concentrations but inhibits at low substrate concentrations [7].

We have shown in a previous report that EDAC, a water soluble carbodiimide, reacts with a carboxyl group outside the immediate vicinity of the catalytic site and prevents acceleration of acetylcholine hydrolysis by organic and inorganic ligands [8]. Acceleration of the decarbamylation reactions by these ligands is also blocked after EDAC-modification (unpublished results). This study on the effect of acceleratory ligands on carbamylation by NPDC of the esteratic site [16] of the EDAC-modified enzyme has indicated that the same allosteric site which is responsible for modulating increased reactivity of the esteratic site in 'deacylation' reactions (decarbamylation and deacetylation) also functions in acceleration of carbamylation of the esteratic site, since acceleration by gallamine and decamethonium is greatly reduced after EDAC-modification (table 1). The molecular mechanism by which this modulation occurs is still unknown at present.

There is no obvious relationship between the computed thermodynamic parameters of the carbamylation by NPDC in table 2 ( $\Delta H^\ddagger$  and  $\Delta S^\ddagger$ ) and the kinetic effects of the ligands on this reaction. Thus, neither acceleration nor inhibition of the reaction is associated with a marked change in either enthalpy or entropy of the control reaction, which have the values 12.8 kcal/mole and  $-26$  e.u., respectively. These

values are similar to those previously determined for the decarbamylation reaction under similar conditions (namely, 13.2 kcal/mole and  $-34$  e.u.) [13]. However, whereas the decarbamylation reaction showed ligand sensitive discontinuous Arrhenius plots, which were interpreted as indicating the occurrence of different temperature and ligand dependent forms of the dimethylcarbamylated enzyme [13], in the present study the Arrhenius type plots in the presence or absence of ligands are linear. This suggests that the conformation of acetylcholinesterase in the 'acylated' form is a more sensitive probe of ligand-induced conformational changes than is the free enzyme. However, the possibility that the NPDC structure stabilize a single conformation of the enzyme cannot be eliminated. Main [17] has found evidence for the occurrence of multiple reversible forms of erythrocyte acetylcholinesterase in studies on the inhibition of the free enzyme, although demonstration of this effect was dependent on the structure of the irreversible inhibitor used.

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