Biochemical Pharmacology, Vol. 34, No. 4, pp. 533–538, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ACTIVATION AND INACTIVATION OF BOVINE CAUDATE ACETYLCHOLINESTERASE BY TRIVALENT CATIONS

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(*Received* 19 *December* 1983; *accepted* 13 *June* 1984)

Abstract—Kinetic analysis of the interaction of trivalent cations with mammalian brain acetylcholinesterase revealed at least three distinct concentration-dependent effects on enzyme activity. Acetylcholinesterase was purified from bovine caudate nucleus by affinity chromatography to a specific activity of 1.1 mmoles acetylthiocholine · hr^{-1} · (mg protein)$^{-1}$. The cations studied included the chloride salts of lanthanum, terbium, yttrium and scandium in low and high ionic strength buffers (2 mM Pipes ± 0.1 M NaCl). At low ionic strength, high affinity noncompetitive or allosteric activation was observed at very low cation concentrations (1–10 μM); at higher concentrations (50–200 μM) these cations were noncompetitive inhibitors; and at 200–500 μM they exerted a mixed competitive–noncompetitive inhibition. Activation by low cation concentrations was not evident in high ionic strength buffers, while enzyme inhibition by all the trivalent cations was similar at low and high ionic strength. Inhibition by all of the multivalent cations was fully reversed by a 10-fold excess of EDTA or by a 100-fold dilution of the inhibited enzyme. The water-soluble carboxyl group affinity reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, was shown to specifically block the activating effect of the multivalent cations supporting the suggestion that the β- or "activator" peripheral anionic sites (P_1) involve a carboxyl group outside the enzyme active site.

A detailed knowledge of the surface of the enzyme, both in the direct vicinity of the active site and at neighboring regions that may influence the active site, is important for the rational design of inhibitors, reactivators, affinity reagents and other ligands for acetylcholinesterase (AChE; EC 3.1.1.7). It is well-established that the surface topography of AChE includes a number of anionic sites separate from the catalytic site. Some of these "peripheral" anionic sites have been identified by their ligand-binding properties, largely in studies utilizing erythrocyte enzyme or enzyme from fish electric organs [1, 2]. The peripheral anionic sites are of interest primarily because the interaction of a variety of cationic ligands, including cholinergic receptor ligands, with these sites can modify the kinetic properties of the enzyme. Wilson and Silman [3], for example, demonstrated that binding of quaternary ammonium ligands to peripheral anionic sites can block arsenite inhibition of the enzyme, and the action of divalent cations as "noncompetitive" or allosteric activators of AChE has been known for many years [4–6].

We proposed several years ago [7–9] that cations of the lanthanide series may be useful probes of the general topography of AChE and specifically good probes for studying peripheral anionic sites. Many of these elements possess physicochemical characteristics that are readily quantitated [10], and nearly all have a hydrated ionic radius very similar to that of the physiological cations that are known to influence and possibly regulate enzyme activity. It was shown previously that the trivalent cations Y^{3+} and Sc^{3+} are noncompetitive inhibitors of electric organ AChE [11]. Slow, irreversible inactivation of AChE by divalent transition metal ions and by the trivalent cation La^{3+} was demonstrated by Tomlinson *et al.* [12, 13] with electric eel enzyme. The fluorescence of bound terbium by energy transfer from aromatic amino acids has been used as a probe for calcium binding sites in a variety of biological preparations [14] and, recently in this laboratory, as a probe of peripheral anionic "activator" sites in rat brain AChE [15]. That study demonstrated a heterogeneous population of Tb^{3+} binding sites, consistent with multiple, concentration-dependent effects of Tb^{3+} on enzyme activity.

The present study was undertaken to investigate the interaction of a series of trivalent cations with AChE. These cations include the lanthanides, lanthanum and terbium, and the "honorary lanthanides," scandium and yttrium, so-called because of their physicochemical similarities to La^{3+}. In addition, many of the technical difficulties encountered with these cations in physiological media were defined and accommodated for in *in vitro* enzyme assays.

MATERIALS AND METHODS

Preparation of bovine brain AChE. Whole calf brains were obtained fresh from a nearby slaughterhouse and transported on ice to the laboratory where the caudate nucleii were dissected and stored at −70° until used for extraction and purification of AChE. Membrane-bound AChE was partially purified by the procedures of Rakonczay *et al.* [16]

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which were developed for purification of rat brain enzyme. Briefly, the caudate nucleii were homogenized in 10 ml per caudate of saline buffer (12.5 mM sodium phosphate, pH 7.2, 0.4 M NaCl), and the suspension was centrifuged at 53,000 *g* for 2 hr. The rehomogenized pellet was diluted with a 9-fold excess of buffer containing 0.55% Triton X-100, stirred for 30 min, and centrifuged as above. The solubilized enzyme was then absorbed on a Concanavalin A-Sepharose 4B column and eluted with 0.5 M α-methyl-D-mannoside (recovery ≃ 50%). This partially purified enzyme preparation, with a specific activity of about 1200 μmoles·hr^{-1}·(mg protein)$^{-1}$, was utilized for the present studies. Protein content was measured by the procedure of Wang and Smith [17] which excludes interference by Triton X-100.

Quantitation of AChE activity. Enzyme activity was assayed by the Ellman procedure [18] in 2 mM Pipes (piperazine-*N*,*N*′-bis 2-ethanesulfonic acid), pH 6.8, to optimize solubility. Experiments were carried out in both low and high ionic strength buffers (± 0.1 M NaCl), as some specific metal ion effects may be nonselectively masked at high ionic strength. Controls demonstrated optimum enzyme activity in the high ionic strength media. K_{Mapp} and V_{max} were determined from double-reciprocal plots of reaction rate as a function of substrate concentration for acetylthiocholine (ASCh) concentrations of 0.05 to 1.2 mM. The data were analyzed and statistical parameters were calculated by linear regression analysis on a PDP 11/23 computer (Digital Equipment Corp., Maynard, MA).

Carbodiimide modification of AChE. The water-soluble carboxyl group affinity reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), was prepared in 2 mM Pipes buffer, pH 6.8. The slightly lower pH should favor the reaction of EDAC with carboxyl groups [19]. Reversible and irreversible effects of EDAC on enzyme activity were assayed as follows: (1) to measure reversible effects, the reaction was carried out in 1 mM EDAC, 2 mM Pipes buffer, 0.05 to 1.2 mM ASCh and 0.33 mM 5,5-dithio-2-bis-nitrobenzoate (DTNB); (2) to measure irreversible effects of the carbodiimide, the enzyme was pretreated with 1 mM EDAC, and ASCh hydrolysis was measured in the same reaction buffer as above but without EDAC.

Chemicals and reagents. Scandium chloride (99.9%), yttrium chloride (99.9%) and lanthanum chloride (99·9%) were purchased from K & K Biochemicals, Plainview, NY; terbium chloride was from Alfa Ventron, Danvers, MA. All other compounds were purchased from the Sigma Chemical Co., St. Louis, MO.

RESULTS

The data in Table 1 are the V_{max} and K_{Mapp} values, for experiments with $LaCl_3$ and $TbCl_3$, determined from double-reciprocal plots of substrate–activity curves. Representative plots for $LaCl_3$ in low ionic strength and $TbCl_3$ in high ionic strength buffer are shown in panels A and B of Fig. 1. In low ionic strength (μ) buffer (2 mM Pipes), a high affinity "noncompetitive" or allosteric activation was

Table 1. V_{max} and K_{Mapp} values for calf caudate AChE: Effects of $LaCl_3$ and $TbCl_3$ in low and high ionic strength buffers*

Cation concn (μM)	V_{max} [μmoles·hr^{-1}·(mg protein)$^{-1}$]				K_{Mapp} (mM)			
	$LaCl_3$		$TbCl_3$		$LaCl_3$		$TbCl_3$	
	Low μ†	High μ	Low μ	High μ	Low μ	High μ	Low μ	High μ
0	404 ± 36	626 ± 42	306 ± 30	418 ± 14	0.18 ± 0.04	0.23 ± 0.12	0.10 ± 0.03	0.10 ± 0.02
1	478 ± 26	512 ± 3	362 ± 18	417 ± 18	0.22 ± 0.08	0.13 ± 0.10	0.07 ± 0.02	0.20 ± 0.07
10	506 ± 20	414 ± 12	370 ± 14	453 ± 22	0.15 ± 0.03	0.07 ± 0.01	0.05 ± 0.04	0.11 ± 0.05
50	375 ± 30	406 ± 40	330 ± 38	339 ± 15	0.10 ± 0.03	0.28 ± 0.10	0.11 ± 0.05	0.18 ± 0.10
100	386 ± 21	319 ± 11	233 ± 31	228 ± 16	0.19 ± 0.04	0.17 ± 0.04	0.12 ± 0.03	0.16 ± 0.03
200	189 ± 42	293 ± 18	263 ± 33	273 ± 31	0.18 ± 0.04	0.28 ± 0.07	0.73 ± 0.15	0.68 ± 0.30
500	139 ± 35	246 ± 13	206 ± 29	218 ± 25	1.03 ± 0.56	2.74 ± 1.44	0.92 ± 0.37	0.42 ± 0.11

* Values for maximum enzyme activity (V_{max}) and apparent Michaelis constant (K_{Mapp}) were determined by Lineweaver–Burk plots with 0.05 to 2.0 mM ASCh as substrate, using a Fortran IV computer program on a PDP 11/23. Experimental data are expressed as the mean ± S.E. of between three and five kinetic assays.

† Experiments were run in buffers of low μ, i.e. low ionic strength (2 mM, pH 6.8), and of high ionic strength (with 0.1 M NaCl added).

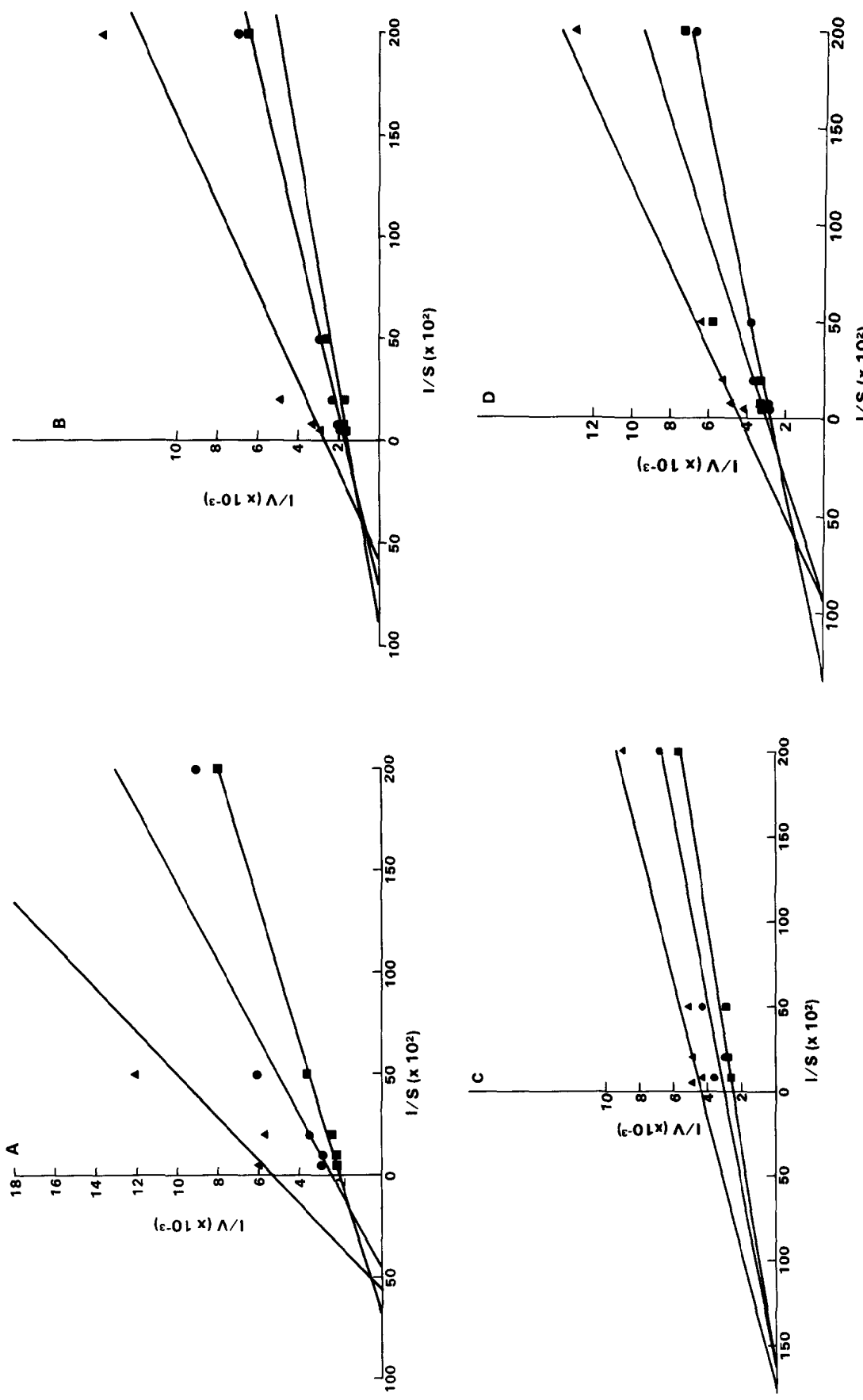

Fig. 1. Representative double-reciprocal plots of enzyme activity vs substrate concentration demonstrating the effects of trivalent cations on the hydrolysis of ASCh by partially-purified bovine caudate AChE. Key: 1 A: (●) control; (■) 10 μM La^{3+}; (▲) 200 μM La^{3+}; all in low ionic strength buffer, 2 mM Pipes, pH 6.8. 1B: (●) control (■) 10 μM Tb^{3+}; (▲) 100 μM Tb^{3+}; all in high ionic strength buffer, 2 mM Pipes, 0.1 M NaCl, pH 6.8. 1C: (●) control; (■) 10 μM Sc^{3+} (▲) 50 μM SC^{3+}; all in low ionic strength buffer. 1D: (●) control; (■) 10 μM Y^{3+}; (▲) 50 μM Y^{3+}; all in high ionic strength buffer. The values are the mean ± S.E. of three kinetic assays; in all cases, the standard errors fall within the area of the symbol used to denote a point.

observed at low La^{3+} and Tb^{3+} concentrations (1–10 μM). At higher concentrations (50–200 μM), these cations were noncompetitive enzyme inhibitors; at 200–500 μM they exerted a mixed competitive–noncompetitive inhibition. In high ionic strength buffer (2 mM Pipes + 0.1M NaCl), the activation at low cation concentrations was not observed. The effects of higher cation concentrations, however, were the same as those seen in low ionic strength media. Tb^{3+} appears to have a higher affinity than La^{3+} for the catalytic site, for at 200 μM it produced a significant increase in the K_{Mapp}, an effect observed only at 500 μM La^{3+}.

Table 2 exhibits data for the trivalent cations Sc^{3+} and Y^{3+}. V_{max} and K_{Mapp} values were determined from double-reciprocal plots such as that shown in Fig. 1C for $ScCl_3$ in low ionic strength and in Fig. 1D for YCl_3 in high ionic strength buffer. Again, in low ionic strength, 1–10 μM Sc^{3+} increased the activity of AChE with no change in the affinity of the catalytic site for the substrate ASCh. However, there was no significant activation by Y^{3+}. Higher concentrations of both cations produced an apparent noncompetitive inhibition of enzyme activity; and at 500 μM both cations exerted a mixed competitive–noncompetitive inhibition. Affinity of the cations for the active or catalytic site is, thus: $Y^{3+} > Tb^{3+} > La^{3+} > Sc^{3+}$. Tb^{3+} and Y^{3+}, the trivalent cations with the highest affinity for the catalytic site, have ionic radii close to 0.90 Å, slightly smaller than Ca^{2+} (0.99 Å) but considerably larger than Sc^{3+} (0.73 Å).

Reversal of inhibition by 100 and 500 μM concentrations of each cation was measured by adding a 10-fold excess of EDTA, a cation chelating agent, to the incubating media. Assays were run at both low and high ionic strength and demonstrated that enzyme inhibition could be reversed completely by chelation. Dilution experiments, similarly, demonstrated complete reversal of the multivalent cation effects in the concentration range studied.

The results of enzyme modification by EDAC are shown in Table 3. The water-soluble carboxyl group affinity reagent exerted a small but significant effect on the catalytic activity of AChE. When assayed with increasing substrate concentrations (0.05 to 1.2 mM), in the presence of a fixed EDAC concentration, reversible competitive inhibition of enzyme activity was evident. If the enzyme was incubated for 4 hr with 1 mM EDAC (5°), following the procedure of Roufogalis and Wickson [20], and aliquots were removed for assay to a buffer-substrate reagent without EDAC, irreversible enzyme inhibition was evident (17 ± 8% decrease in V_{max}). The carbodiimide-treated enzyme was not activated by 1 mM Ca^{2+} or by a 10 μM concentration of the trivalent cations. A minimum of 4 hr treatment of enzyme with EDAC was required to abolish the activation by cations, and, as reported by Roufogalis and Wickson [20], this effect of EDAC increased progressively with time.

Table 2. V_{max} and K_{Mapp} values for calf caudate AChE: effects of $ScCl_3$ and YCl_3 in low and high ionic strength buffers*

Cation concn (μM)	V_{max} [μmoles · hr^{-1} · (mg protein)$^{-1}$]				K_{Mapp} (mM)			
	$ScCl_3$		YCl_3		$ScCl_3$		YCl_3	
	Low μ†	High μ	Low μ	High μ	Low μ	High μ	Low μ	High μ
0	323 ± 38	442 ± 19	256 ± 2	351 ± 19	0.06 ± 0.03	0.07 ± 0.01	0.06 ± 0.002	0.07 ± 0.02
1	382 ± 7	351 ± 18	257 ± 18	348 ± 9	0.09 ± 0.01	0.06 ± 0.02	0.07 ± 0.03	0.07 ± 0.01
10	468 ± 57	401 ± 29	279 ± 13	331 ± 29	0.23 ± 0.09	0.06 ± 0.02	0.06 ± 0.02	0.11 ± 0.04
50	238 ± 8	373 ± 51	271 ± 17	295 ± 23	0.05 ± 0.01	0.16 ± 0.08	0.12 ± 0.03	0.09 ± 0.03
100	249 ± 3	341 ± 41	198 ± 16	223 ± 10	0.09 ± 0.01	0.11 ± 0.05	0.09 ± 0.04	0.11 ± 0.02
200	229 ± 22	309 ± 54	126 ± 7	170 ± 6	0.08 ± 0.03	0.13 ± 0.08	1.10 ± 0.67	0.20 ± 0.03
500	167 ± 13	177 ± 8	156 ± 12	185 ± 13	0.26 ± 0.08	0.17 ± 0.05	2.00 ± 0.41	0.90 ± 0.16

* Values for maximum activity (V_{max}) and an apparent Michaelis constant (K_{Mapp}) were determined as in Table 1, and are expressed as the mean ± S.E. of between three and five experiments.

† Experiments were run in low and high ionic strength (μ) Pipes buffer as in Table 1.

DISCUSSION

Several technical problems may be encountered in studying the effects of lanthanides and other multi-

Table 3. Carbodiimide modification of anionic site reactivity of bovine caudate AChE*

Sample	K_{Mapp} (mM)	V_{max} [μmoles·hr^{-1}·(mg protein)$^{-1}$]
(A) Reversible effects†		
Control	0.06 ± 0.01	213.5 ± 13.3
0.02 mM EDAC	0.12 ± 0.01	221.2 ± 6.3
0.05 mM EDAC	0.31 ± 0.04	188.6 ± 9.7
(B) Irreversible effects‡		
Control	0.06 ± 0.02	636.6 ± 65.8
1 mM EDAC +	0.05 ± 0.02	484.5 ± 45.9
1 mM Ca^{2+}	0.05 ± 0.01	520.2 ± 34.0
10 μM La^{3+}	0.08 ± 0.02	485.0 ± 44.9
10 μM Tb^{3+}	0.04 ± 0.02	494.8 ± 63.6
10 μM Sc^{3+}	0.04 ± 0.02	477.3 ± 26.6
10 μM Y^{3+}	0.06 ± 0.02	484.0 ± 27.7

* Values of each parameter are expressed as the mean ± S.E. of five experiments for each condition. EDAC = 1-ethyl-3(3-dimethylaminopropyl) carbodiimide.

† The enzyme reactions were carried out in the presence of 1 mM EDAC, 2 mM Pipes buffer, pH 6.8, with 0.05 to 1.2 mM ASCh and 0.33 mM DTNB.

‡ Enzyme was pretreated with 1 mM EDAC, and ASCh hydrolysis was measured in the same reaction buffer as above but without EDAC. The effect of calcium and trivalent cations were assayed on pretreated enzyme by adding the cations to the reaction buffer.

valent cations in biochemical systems. Using $EuCl_3$, Ellis and Morrison [21] demonstrated that the time-dependent loss of the ability of dilute (μM) solutions to inhibit creatinine kinase is due primarily to absorption of Eu^{3+} onto the walls of containers. Mellanby and Thompson [22] tried to overcome the uncertainties of aqueous $LaCl_3$ solutions by calculating the concentration of free La^{3+} in acetate buffer according to the coordination complexes measured by Sonesson [23].

Our earlier studies with La^{3+} and electric eel enzyme [9] indicated that low pH and acid-washed, phosphate-free glassware enhanced the apparent solubility of $LaCl_3$ in high ionic strength solutions. Our experience with the present studies, however, confirms the findings of Tomlinson *et al.* [13] that the solutions used in those earlier studies probably contained fine precipitates and, thus, much lower concentrations of La^{3+} as the reaction proceeded over time. In addition, such experiments with crude enzyme and intact pieces of tissue may result in nonspecific interactions of La^{3+} concentrations in the experimental solution during the time course of the assay. In that case, the activation evident at low concentrations may, in fact, appear to be delayed [9].

Dos Remedios [14] pointed out that, while many commonly used buffers such as Tris exhibit significant affinities for Ca^{2+} and the lanthanides, other buffers such as Pipes and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) have low affinities for divalent and trivalent cations and are particularly suitable for use with dilute solutions. Freshly prepared 500 μM solutions of $ScCl_3$, YCl_3, $LaCl_3$, and $TbCl_3$ in Pipes buffer, pH 6.8, stored at room temperature, yielded highly reproducible data throughout the course of our studies. On the other hand, solutions of lanthanides at concentrations in the mM range in Tris buffer, pH 8.0, as utilized by Tomlinson *et al.* [13], were frequently observed to contain fine precipitates within minutes after preparation.

At low ionic strength, rapid activation was seen with three of the four cations studied at concentrations three orders of magnitude below the total ionic strength of the buffer. The activation is an apparent allosteric effect and involves no change in the affinity of the catalytic site of AChE for ASCh. At higher concentrations, still well below the ionic strength of the buffer, a competitive effect becomes evident. In high ionic strength buffer, the cations are exclusively enzyme inhibitors, exhibiting largely noncompetitive inhibition at lower concentrations and mixed competitive-noncompetitive inhibition at high concentrations. The enzyme preparation used in this study was about 100 to 200-fold less pure than homogeneous bovine caudate AChE. Thus, the possibility of nonenzymic binding sites for multivalent cations cannot be ruled out. The observation, however, that noncompetitive inactivation persists at high ionic strength indicates that peripheral ligands may bind to the enzyme in physiological milieu and may influence enzyme reactivity *in situ* under those conditions.

Several investigators have shown that activation of AChE by Ca^{2+} is an allosteric effect and that Ca^{2+} binds to peripheral anionic sites on the enzyme [2, 5, 6]. A more hydrophobic class of peripheral anionic sites has been identified and may be one site of action of the neurotoxic cation Al^{3+} [11]. Tomlinson *et al.* [13] described a simple model of enzyme reactivity involving active, activated and unreactive states of the enzyme and suggested that the distribution of enzyme among these forms is dependent upon the binding of cations to distinct activator sites effectively masked in high ionic strength solutions. Our results are consistent with that model and reinforce the possibility that enzyme reactivity *in situ* may be modulated by a variety of metal cations.

The Ca^{2+}-binding activator sites, labeled β- by Roufogalis and Quist [2] and P_1 by Rosenberry [24], quite likely involve a carboxyl group. In erythrocyte enzyme, the allosteric activation by Ca^{2+} was found to be blocked by the water-soluble carbodiimide EDAC [20, 25]. As shown above, this carboxyl group modifying reagent does indeed block all of the activating effects observed with multivalent cations under conditions in which the reagent itself exerts only a minimal inhibition of enzyme activity. Thus, in the 1–10 μM concentration range, the multivalent cations studied bind largely to β- or "activator" peripheral anionic sites and binding involves a carboxyl group that can be blocked by carbodiimide. The lanthanides should, as suggested, be meaningful physicochemical probes of these binding sites, and the present data have established a useful baseline for the fluorescence studies now in progress.

Acknowledgements—Supported by the U.S. Army Research Office (DAAG29-K-89-0042), the Biomedical Research Support Grant at Boston University School of Medicine, and the Center for Brain Sciences, Cambridge, MA. We are grateful to Mr. Robert D. MacCallum for skillful technical assistance.

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