Lowry, O. H., Rosebrough, M. J., Farr, A., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Muhn, P., & Hucho, F. (1983) *Biochemistry 22*, 421–425.
Muhn, P., Lauffer, L., & Hucho, F. (1983) in *Toxins as Tools in Neurochemistry* (Hucho, F., & Ovchinnikov, Yu, Eds.) de Gruyter, Berlin.
Neubig, R. R., & Cohen, J. B. (1980) *Biochemistry 19*, 2770–2779.
Neubig, R. R., Boyd, N. D., & Cohen, J. B. (1982) *Biochemistry 21*, 3460–3467.
Oswald, R., & Changeux, J.-P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3925–3929.
Oswald, R., Heidmann, T., & Changeux, J.-P. (1983) *Biochemistry 22*, 3128–3136.
Schiebler, W., & Hucho, F. (1978) *Eur. J. Biochem. 85*, 55–63.

Activation of Acetylcholinesterase by Monovalent (Na^+, K^+) and Divalent (Ca^{2+}, Mg^{2+}) Cations†

Peter Hofer, Urs Peter Fringeli,* and Wolfgang Heinrich Hopff

ABSTRACT: The activation of acetylcholinesterase [EC 3.1.1.7 (AChE)] by monovalent and divalent metal ions has been investigated by kinetic experiments under steady-state conditions (pH-stat method). It has been shown that at low ionic strength the enhancement of the activity by both monovalent and divalent metal ions can be explained as an electrostatic effect. Thereby, enhancement of the concentration of monovalent metal ions (Na^+ and K^+) acts by reducing the penetration depth of the electric field of carboxylate groups located at the active center whereby divalent metal ions (Ca^{2+} and Mg^{2+}) act by a complex formation with these charged groups.

It has been known for many years that the enzymatic activity of acetylcholinesterase [EC 3.1.1.7 (AChE)][1] is enhanced by alkaline metal ions (Na^+ and K^+) and alkaline-earth metal ions (Ca^{2+} and Mg^{2+}) whereby the activation caused by the second group is much greater than that caused by the first group (Nachmansohn, 1940). There are at least three explanations for this activating effect: (1) ionic strength effects according to the theories of Brönsted–Bjerrum and Debye–Hückel; (2) conformational changes as a result of a peripheral site occupation by the metal ions; (3) specific binding of the metal ion to the anionic subsite of the active centre.

Roufogalis & Wickson (1973, 1975) have shown that the activation by calcium ions is inhibited by a treatment of the enzyme with carboxyl group reagents such as carbodiimide. They interpreted this observation as the inhibition of an allosteric effect.

Nolte et al. (1980) explained the influence of the sodium concentration on the association rate constant of the cationic ligand *N*-methylacridinium with AChE as an ionic strength effect, which can be described by the Brönsted–Debye–Hückel theory.

Smissaert (1981) interpreted his results as a "specific salt effect". Thereby, the association of the Na^+ ions with the anionic subsite of the catalytic center reduces the reactivity (k_{cat}/K_m) of the enzyme.

Tomlinson et al. (1981) explained the activating effect of the divalent metal ions Ca^{2+} and Mg^{2+} as a conformational change which is induced by the occupation of a peripheral site of the enzyme by these ions. A voluminous literature deals with "peripheral anionic sites" of AChE. For a review the reader is referred to Rosenberry (1975), Bolger & Taylor (1979), and Berman et al. (1981).

In this work we want to examine whether the activation of AChE by mono- and divalent metal ions can be explained only by ionic strength effects or whether additional mechanisms have to be taken into consideration.

Materials and Methods

Enzyme Purification. AChE from the electric organ of *Torpedo marmorata* was purified by affinity chromatography as described by Hopff (1976). Density gradient centrifugation revealed that the preparation contained 50% of the globular 11S form and 50% of the asymmetric forms (~10% of 17S, ~25% of 13.3S, and ~15% of 9S).

Enzyme Assay (pH-Stat Method). In a total volume of 5 mL, the mixture contained 10^{-3} M acetylcholine iodide (AChI) or bromide and various concentrations of the chlorides of the salts of mono- and divalent metal ions. The reaction was started by adding 5 μL of enzyme solution (~1 IU).[1] To keep the pH constant 0.01 M NaOH was added by the use of a pH meter equipped with an impulsomat and a dosimat. The temperature was 25 °C, and the reaction mixture was kept under nitrogen. For activity determinations at varied salt concentrations the pH was kept at 7.4. If the activity at varied pH value was measured, the pH dependency of the pH-stat method had to be taken into consideration. At low pH (<6) the protonation of the acetic acid increases, thus leading to a reduced demand for NaOH. At high pH values (>9.5) the amount of NaOH is too large because of the increased deprotonation of the choline groups.

Results and Discussion

Effect of Monovalent Cations (Na^+ and K^+) on the Enzymatic Activity. Figure 1 shows the results of activity measurements at different sodium concentrations in the form log

† From the Laboratory for Physical Chemistry, Swiss Federal Institute of Technology, CH-8092 Zurich, Switzerland (P.H. and U.P.F.), and the Institute of Pharmacology, University of Zurich, CH-8006 Zurich, Switzerland (W.H.H.). *Received August 10, 1983; revised manuscript received November 23, 1983.* This work was supported by the Swiss National Science Foundation (Project 3.549-3.79) and by the Emil Barell Foundation (Hoffmann-La Roche, Project 127).

[1] Abbreviations: AChE, acetylcholinesterase (EC 3.1.1.7); AChI, acetylcholine iodide; 1 IU, 1 international unit = 1 μmol of product produced/min.

0006-2960/84/0423-2730$01.50/0

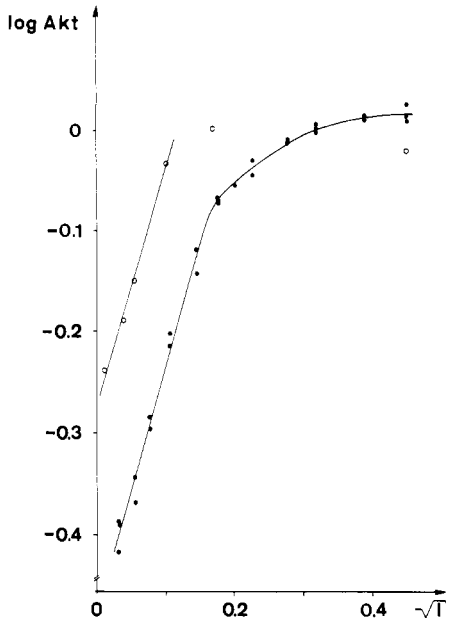

FIGURE 1: Dependence of the enzymatic activity on the ionic strength. (○) Results of Tomlinson et al. (1981). (●) Our results (pH-stat method, T = 25 °C, pH 7.4, acetylcholine concentration = 10^{-3} M, I in mol/L).

(Act) = $f(I^{1/2})$ (I = ionic strength = $1/2\sum c_i z_i^2$ whereby c_i and z_i denote the concentration and the charge of the ion i). In this experiment the ionic strength was identical with the sodium chloride concentration. By use of potassium chloride instead of sodium chloride, identical results have been obtained, thus showing that the increase of activity (Figure 1) is caused by an increased ionic strength and not by a specific sodium effect.

Such a dependency of the reaction rate and the ionic strength can be described by the theories of Brönsted–Bjerrum and Debye–Hückel. According to them the rate constant of a reaction between two charged particles depends on the ionic strength as follows (Kortüm, 1972):

$$\log k = \log k_0 + \frac{2AZ_1Z_2I^{1/2}}{1 + BaI^{1/2}} \qquad (1)$$

k_0 = the rate constant at ionic strength = 0, Z_1 and Z_2 = the charges of the two reactants, A and B are constants, A = 0.509 and B = 0.33 (T = 298 K, ϵ = 80.3), a = the minimal distance (Å) of the two charges, and I = the ionic strength (mol/L). The activity of AChE is limited by the slowest step in the enzymatic hydrolysis of acetylcholine S^+ to choline Ch^+ and acetic acid Ac^-.

$$E + S^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES^+ \xrightarrow{k_2\,Ch^+} E\text{–}Ac \xrightarrow[H_2O]{k_3} E + Ac^- + H^+$$

(E stands for the free enzyme and E–Ac for the acetylated enzyme.)

Different groups (Wilson & Cabib, 1956; Krupka, 1964; Froede & Wilson, 1980) have found that the deacetylation (k_3) is the rate-limiting step, i.e., $k_2 > k_3$. That means that the ionic strength dependency of the turnover number (k_{cat}) and the activity (Act) is comparable to that of the deacetylation step.

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3} \simeq k_3$$

$$\text{Act} = k_{cat}[E]_t \simeq k_3[E]_t$$

where $[E]_t$ = total enzyme concentration

$$\log (\text{Act}) = \log (\text{Act})_0 + \frac{2AZ_1Z_2I^{1/2}}{1 + BaI^{1/2}} \qquad (2)$$

At low ionic strength the following linearization may be applied:

$$\log (\text{Act}) = \log (\text{Act})_0 + 2AZ_1Z_2I^{1/2} \qquad (3)$$

At high ionic strength the Debye–Hückel expression for the activity coefficients is extended by a linear term (Kortüm, 1972) which also leads to a linear term in eq 2:

$$\log (\text{Act}) = \log (\text{Act})_0 + \frac{2AZ_1Z_2I^{1/2}}{1 + BaI_{1/2}} + CI \qquad (4)$$

On the basis of eq 2 two conclusions can be drawn from Figure 1: (1) There are two charged particles reacting in the rate-limiting step, i.e., in the deacetylation. (2) The positive slope of the graph indicates that the charges of these two particles are of the same sign.

According to Nolte et al. (1980) the net charge Z_A of the active site amounts to $-6.3e_0$ (e_0 = 1.6×10^{-19} C). The deacetylation occurs under the electrostatic influence of these negative charges. The fact that the two reactants are of the same sign indicates that the acetylated enzyme reacts with a negative charged particle. This is the case with a base-catalyzed ester hydrolysis:

$$E(Z_A)\text{–}Ac + OH^- \rightarrow E(Z_A)\text{–}OH + Ac^-$$

$E(Z_A)$–OH = AChE, $E(Z_A)$–Ac = acetylated AChE, and Z_A = the net charge of the active center. Before the Brönsted–Debye–Hückel theory is applied to this reaction, it should be remembered that this theory describes the reaction between two charged particles. The base-catalyzed deacetylation of AChE, however, is a reaction between the negative hydroxyl ion and a positive polarized carbonyl group whereby the latter is surrounded by about six negative charged carboxyl groups (Nolte et al., 1980). The obtained value for the effective charge Z_E shows the effect of these carboxyl groups on the hydroxyl ion and is probably smaller than the total charge Z_A of the active site.

Up to an ionic strength of 25 mM the data (Figure 1) can be described very well by the linearized Brönsted–Bjerrum expression (eq 3). The estimated effective charge amounts to $Z_E = (-2.3 \pm 0.1)e_0$ (indicated errors correspond to the 90% confidence limits). Measurements by Tomlinson et al. (1981) lead to practically the same result.

Thus, according to the theories of Brönsted–Bjerrum and Debye–Hückel, the reason for the enhancement of the activity by sodium and potassium ions is a reduction of the range of the electric field of the carboxylate groups, which reduces the electrostatic repulsion between these groups and the hydroxyl ions. At ionic strengths above 20 mM either the nonlinearized or the extended Brönsted–Bjerrum equation might be applied (eq 3 and 4, respectively).

The agreement of the least-squares fits and the data in either case is not very good (Figure 2). For the (a) nonlinearized Brönsted–Bjerrum equation (eq 2), the fitted parameters are $Z_E = (-8.6 \pm 3.2)e_0$ a = 33 ± 11 Å. Parameter a is much too big to be realistic. A reaction between a hydroxyl ion and an acetyl group at a distance of 33 Å is hardly possible. This minimal distance is also too big in comparison with the steric extensions of the active center as estimated by Hopff (1976). (b) For the extended Brönsted–Bjerrum equation (eq 4), the fitted parameters are $Z_E = (-4.5 \pm 1.2)e_0$, a = 12 ± 6 Å, and $C = -1.0 \pm 0.5$ M^{-1}. As the introduction of the linear term is purely arbitrary and this constant C has no meaning in the molecular model, the rest of the parameters too represent

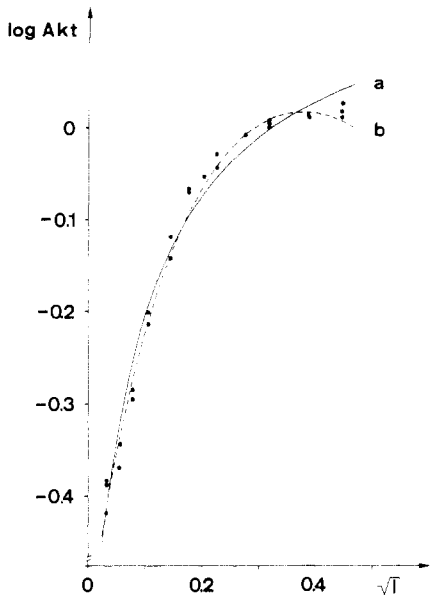

FIGURE 2: Dependence of the enzymatic activity on the ionic strength. Least-squares fits on the base of the nonlinearized (a) (eq 2) and of the extended (b) (eq 4) Brönsted–Bjerrum equation (I in mol/L).

merely fit parameters of little meaning. The appearance of a maximum in the fitted curve which is not seen in our data shows that this least-squares fit is not very likely.

The failure of the Brönsted–Bjerrum theory at high ionic strength may be due to the following reasons: (1) The assumptions for applying this theory are not fulfilled. (2) The range of application of the Debye–Hückel theory is exceeded. (3) The charge and the conformation (eventually causing subunit interactions) of the AChE may depend on the ionic strength. Such a change of a reactant during the measurement may influence the results.

(Case 1) As mentioned above, the Brönsted–Bjerrum theory has been developed for reactions between charged particles. With the acetylated AChE, which is one reactant at the deacetylation, the reacting group and the charged group are not identical. However, the application of the Brönsted–Bjerrum theory is obviously an efficient means for interpreting the experimental data at low ionic strengths.

(Case 2) Concerning the range of application of the Debye–Hückel theory the opinions differ greatly. The concentrations above which the theory is said to fail reach from 1 to 100 mM (Frank & Thompson, 1959; Glückauf, 1959). However, the extended theory describes the mean activity coefficients of sodium chloride up to a concentration of ~10 M (Kortüm, 1972).

(Case 3) Polyelectrolytes carry many charges which interact with each other. An increase of the ionic strength will reduce these interactions which lead to a small change of the conformation of the protein. Moreover, in the case of poly(carbonic acids) such a reduced interaction will cause an increased dissociation of the carboxylic groups, i.e., an increased number of charges. Both a conformational change and a change of the net charge might influence the activity of an enzyme. Concerning salt-sensitive conformational effects, there is some evidence from infrared spectroscopic measurements that the enzyme conformation is slightly altered when the ionic strength passes the "discontinuity" in the Brönsted–Bjerrum plot (Figure 1) at ~36 mM ($I^{1/2} \simeq 0.19$). These spectroscopic findings will be published elsewhere.

Effect of Divalent Cations (Ca^{2+} and Mg^{2+}) on the Enzymatic Activity. Figure 3 shows the dependence of the enzymatic activity of AChE on the ionic strength whereby at a

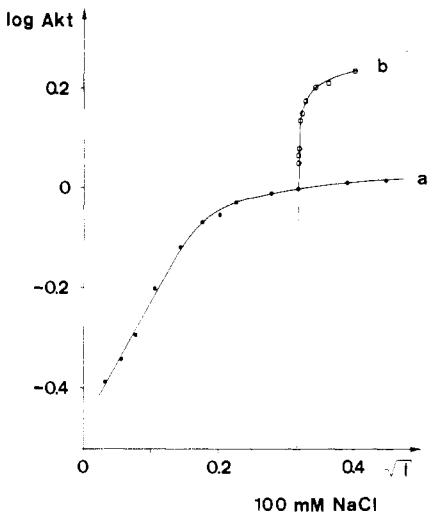

FIGURE 3: Dependence of the enzymatic activity on the ionic strength I. (a) I = [NaCl]; (b) I = 0.1 M NaCl + 3[$CaCl_2$] (pH-stat method, T = 25 °C, pH 7.4, acetylcholine concentration = 10^{-3} M, I in mol/L).

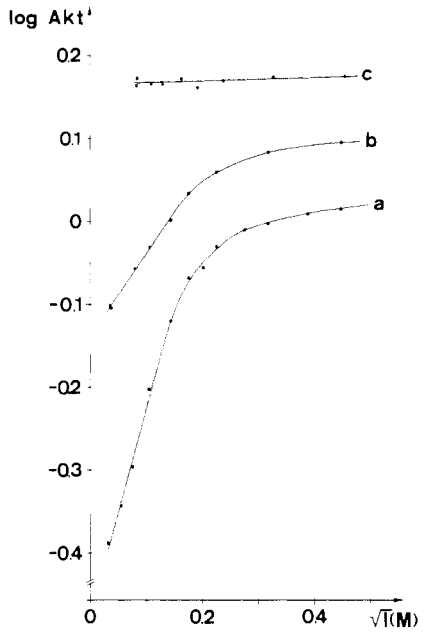

FIGURE 4: Dependence of the enzymatic activity on the ionic strength I. (a) Without $CaCl_2$; (b) 0.1 mM $CaCl_2$ and (c) 2 mM $CaCl_2$ have been added (pH-stat method, T = 25 °C, pH 7.4, acetylcholine concentration = 10^{-3} M, $I = {}^1/_2\sum c_i z_i^2$ = [NaCl] + 3[$CaCl_2$]).

NaCl concentration above 100 mM the further increase of the ionic strength was caused by the addition of either NaCl (a) or $CaCl_2$ (b). Obviously the effect of the calcium ions on the esterase activity is different from that of the sodium salt.

More insight is given by the following experiment: At a constant $CaCl_2$ concentration of 0.1 and 2 mM, respectively, the influence of the ionic strength on the enzymatic activity was measured. The ionic strength was varied by the addition of NaCl. The results are shown in Figure 4. As the influence of NaCl on the enzymatic activity up to a concentration of 25 mM can be interpreted in terms of an ionic strength effect (see above), only this linear part has been taken into consideration.

For comparison Figure 4a represents the ionic strength dependence of the activity of AChE in the absence of Ca^{2+} ions. At a $CaCl_2$ concentration of 0.1 mM the slope of the Brönsted–Bjerrum graph (Figure 4b), i.e., the charge of the active site, is reduced by 60%. When the concentration of $CaCl_2$ is increased to 2 mM (Figure 4c), the enzymatic activity

Table I: Dissociation Constants of Different Carbonic Acids with Calcium (Ionic Strength → 0 M)

acetic acid	0.17 M[a]
malonic acid	3.2×10^{-3} M[b]
citric acid	2.1×10^{-5} M[c]

[a] Coleman-Porter & Monk (1952) [see Sillén & Martell (1964, p 365)]. [b] Stock & Davies (1949) [see Sillén & Martell (1964, p 385)]. [c] Davies & Hoyle (1955) [see Sillén & Martell (1964, pp 477 and 478)].

is practically independent of the ionic strength. It is obvious that calcium ions neutralize the negative charges of the active site by a complex formation with the carboxylic groups. This leads to an easier access of the negative charged hydroxyl ions at the deacetylation step and thus enhances the enzymatic activity. Similar results have been obtained for magnesium.

The determined values for the effective charges at $CaCl_2$ concentration of 0.1 and 2 mM amount to

$$Z_{E_1} = (-0.96 \pm 0.05)e_0 \qquad [CaCl_2] = 0.1 \text{ mM}$$

$$Z_{E_2} = (-0.02 \pm 0.02)e_0 \qquad [CaCl_2] = 2 \text{ mM}$$

From these data the complex formation constant K_A of the calcium ions with the binding sites $(-COO^-)_n$ of the active center has been evaluated (total concentration of binding sites = $[(-COO^-)_n]_t$)

$$(-COO^-)_n + Ca^{2+} \rightleftarrows (-COO^-)_nCa^{2+}$$

$$K_A = \frac{[(-COO^-)_nCa^{2+}]}{[(-COO^-)_n][Ca^{2+}]} \frac{f_{(-COO^-)_nCa^{2+}}}{f_{(-COO^-)_n} f_{Ca^{2+}}} \simeq \frac{Z_E - Z_{E_1}}{Z_{E_1} \times 10^{-1} \text{ M}}$$

$[(-COO^-)_n]_t \propto Z_E$, $[(-COO^-)_n] \propto Z_{E_1}$, $[(-COO^-)_nCa^{2+}] \propto (Z_E - Z_{E_1})$, and $[Ca^{2+}] = 10^{-4}$ M $- [(-COO^-)_nCa^{2+}] \approx 10^{-4}$ M. Considering the low concentrations ($[Ca^{2+}] = 10^{-4}$ M, $[(-COO^-)_n] \simeq 10^{-9}$ M) the reactants are assumed to show ideal behavior, i.e., $f_i = 1$. The complex formation constant K_A and the dissociation constant K_D ($=1/K_A$) are calculated to be $K_A = (1.4 \pm 0.4) \times 10^4$ M^{-1} and $K_D = (7.4 \pm 2.4) \times 10^{-5}$ M. The corresponding value of K_D determined by Taylor & Lappi (1975) amounts to $(4.3 \pm 0.4) \times 10^{-4}$ M. When this is compared to our results, one has to consider that these authors measured the association of Ca^{2+} ions with the whole protein surface while with our method only the one with the active site was determined. The density of the carboxylic groups at the active site area probably differs from that of the rest of the protein surface. The great influence of this "carboxylic group density" on the dissociation constant of complexes of Ca^{2+} with carbonic acids is demonstrated in Table I. As concluded from Table I our determined value of K_D suggests that about three carboxylic groups are involved in one calcium-binding site at the active center. At a low ionic strength the enhancement of the enzymatic activity by Ca^{2+} and Mg^{2+} can obviously be explained by a complex formation of these ions with the carboxylic groups of the active center. Thereby, these ions neutralize the negative charge of the latter, which leads to an easier access for the hydroxyl ion. Thus, at low ionic strengths the observed activation can be explained by electrostatic effects, and there is no need to introduce a conformational change to explain the presented data. More recently, however, infrared spectroscopic data revealed a significant change of the protein secondary structure upon concentration modulation of Ca^{2+} ions between 0.25 and 0.75 mM in the presence of 100 mM NaCl (*cf.* Figure 3). These results will be published elsewhere.

Effect of Ionic Strength on the Activity vs. pH Curve. Figure 5 represents the enzymatic activity of AChE at varied pH values, measured at an ionic strength of 0.1 (0.1 M NaCl) (a) and 0.006 M (0.005 M NaCl + 0.001 M AChI) (b). The lowering of the ionic strength leads to a reduced activity of the enzyme and a shifting of the maximum of the activity to higher pH values. At low ionic strength (Figure 5b) the penetration depth of the electric field of the carboxylate groups is increased from about 10 to about 40 Å. This leads to an increased repulsion between the active center and the hydroxyl ion, i.e., to a decreased activity. In addition, this enlarged penetration depth leads to an enlarged interaction between the carboxylate groups, thus leading to an increase of the pK values in question. Such a dependence of the pK values of polycarbonic acids on the ionic strength has been measured for citric acid (Table II).

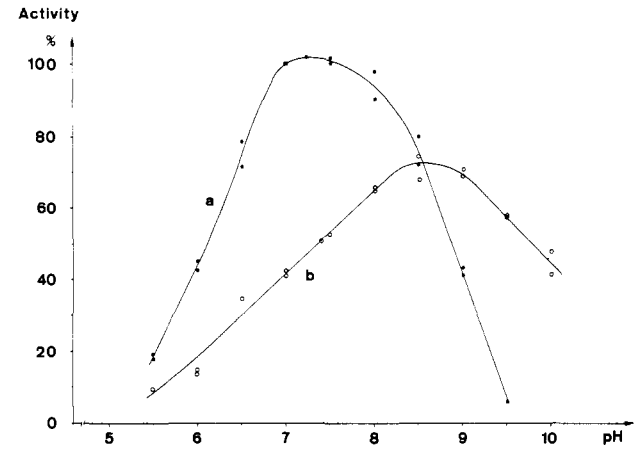

FIGURE 5: Dependence of the enzymatic activity on the pH measured at an ionic strength of 0.1 (a) and 0.006 M (b) (pH-stat method, T = 25 °C, acetylcholine concentration = 10^{-3} M).

Table II: pK Values of Citric Acid at Different Ionic Strengths

ionic strength = 0.15 M (temp = 25 °C)	ionic strength → 0 M (temp = 25 °C)
$pK_1 = 5.62$[a]	$pK_1 = 6.39$[b]
$pK_2 = 4.34$[a]	$pK_2 = 4.73$[c]
$pK_3 = 2.93$[a]	$pK_3 = 3.11$[d]

[a] Li et al. (1959) [see Sillén & Martell (1964 p 477)]. [b] Bates & Pinching (1949) [see Sillén & Martell (1964, p 477)]. [c] Heinz (1951) [see Sillén & Martell (1964, p 478)]. [d] Davies & Hoyle (1953) [see Sillén & Martell (1964, pp 477 and 478)].

At the active center of AChE interactions of not only three but also about six carboxylic groups are expected (Nolte et al., 1980), thereby causing an even enhanced shifting of the pK values. As a consequence of this shifting a certain amount of carboxylic groups remain protonated at relatively high pH values.

This is also illustrated by the following experiment: The dependence of the activity of AChE on the pH was measured in 0.1 M NaCl solution which contained 2 mM $CaCl_2$ (Figure 6a) and in pure 0.1 M NaCl solution (Figure 6b). The main effect of the $CaCl_2$, the increased activity, has been discussed above. But in addition there is a shift of the maximum of the activity to higher pH values in the presence of $CaCl_2$. The reason for this behavior is probably found in an increased deprotonation of the carboxylic groups of the active center at higher pH values which is paralleled by an increased complex formation constant of Ca^{2+} and the active center of AChE. Such a neutralization of the negative charge would lead to the observed enhancement of the enzymatic activity (see above). In 1955 Davies & Hoyle [see Sillén & Martell (1964), pp 477 and 478] indeed found a very strong correlation between the degree of deprotonation of citric acid (H_3L) and the complex formation constant of this acid with Ca^{2+} ions: $K_A(Ca^{2+} +$

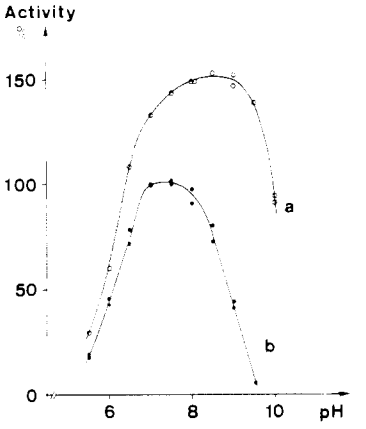

FIGURE 6: Dependence of the enzymatic activity on the pH measured in 0.1 M NaCl with the addition of 2 mM $CaCl_2$ (a) and without $CaCl_2$ (b) (pH-stat method, T = 25 °C, acetylcholine concentration = 10^{-3} M).

$H_2L^- \rightleftarrows CaH_2L^+$) = 12.6 M^{-1}; $K_A(Ca^{2+} + HL^{2-} \rightleftarrows CaHL)$ = 1.23 × 10^3 M^{-1}; $K_A(Ca^{2+} + L^{3-} \rightleftarrows CaL^-)$ = 4.79 × 10^4 M^{-1}. Thus, the observed shift of the maximum of activity induced by Ca^{2+} ions indicates that at a pH value of 7, i.e., at a physiological pH value, there still are protonated carboxylic groups in the active center, a fact which has recently been verified by infrared spectroscopy (unpublished results). From this the following consequences can be drawn: (1) At physiological pH values the carboxylic groups of the active center of AChE are still partly protonated. (2) A change of the "inner electric field" of the protein by changing the ionic strength of the electrolyte leads to a change of the number of charged groups. (3) The activity depends highly on this number of charges. (4) Thus, the inner electric field might be a useful means to control the enzymatic activity of AChE. In this context it should be mentioned that on the basis of infrared–ATR data Fringeli & Hofer (1980) have already postulated an electrostatic model for the regulation of the activity of AChE.

Acknowledgments

We thank M. Fringeli and U. Riester for skillful technical assistance. We also thank B. Hieger and Prof. U. Brodbeck, University of Berne, Switzerland, for the density gradient analysis and valuable discussions. Stimulating discussions with Prof. Hs. H. Günthard, Prof. P. G. Waser, and Dr. P. Thanei are gratefully acknowledged.

Registry No. AChE, 9000-81-1; Na, 7440-23-5; K, 7440-09-7; Ca, 7440-70-2; Mg, 7439-95-4.

References

Berman, H. A., Becktel, W., & Taylor, P. (1981) *Biochemistry 20*, 4803–4810.

Bolger, M. B., & Taylor, P. (1979) *Biochemistry 18*, 3622–3629.

Frank, H. S., & Thompson, P. T. (1959) in *The Structure of Electrolyte Solutions* (Hamer, W. J., Ed.) pp 113–134, Wiley, New York.

Fringeli, U. P., & Hofer, P. (1980) *Neurochem. Int. 2*, 185–192.

Froede, H. C., & Wilson, I. B. (1980) *Neurochem. Int. 2*, 193–197.

Glückauf, E. (1959) in *The Structure of Electrolyte Solutions* (Hamer, W. J., Ed.) pp 97–112, Wiley, New York.

Hopff, W. H. (1976) *Vierteljahrschr. Naturforsch. Ges. Zuerich 121*, 223–260.

Kortüm, G. (1972) *Electrochemistry*, Verlag Chemie, Weinheim/Bergstrasse, West Germany.

Krupka, R. M. (1964) *Biochemistry 3*, 1749–1754.

Nachmansohn, D. (1940) *Nature (London) 145*, 513–514.

Nolte, H. J., Rosenberry, T. L., & Neumann, E. (1980) *Biochemistry 19*, 3705–3711.

Rosenberry, T. L. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 103–218.

Roufogalis, B. D., & Wickson, V. M. (1973) *J. Biol. Chem. 248*, 2254–2256.

Roufogalis, B. D., & Wickson, V. M. (1975) *Mol. Pharmacol. 11*, 352–360.

Sillén, L. G., & Martell, A. E. (1964) *Stability Constants of Metal-Ion Complexes*, Spec. Publ. No. 17, The Chemical Society, Burlington House, London.

Smissaert, H. R. (1981) *Biochem. J. 197*, 163–170.

Taylor, P., & Lappi, S. (1975) *Biochemistry 14*, 1989–1997.

Tomlinson, G., Mutus, B., & McLennan, I. (1981) *Can. J. Biochem. 59*, 728–735.

Wilson, I. B., & Cabib, E. (1956) *J. Am. Chem. Soc. 78*, 202–207.