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High-Concentration Salt Effects in Acetylcholinesterase Reactions

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The influence of inorganic salts on the kinetics of the reactions of acetylcholinesterase with neutral and cationic substrates at 25°C and pH 7.5 in the range of salt concentrations from physiological ionic strength up to nearly saturated solutions has been studied. In both types of substrates, the electrolytes had no influence on the acetylcholinesterase active site acylation and deacylation rate constants—the influence of the salts appeared only in the dissociation constants of the enzyme–substrate (ES) complexes. Two mechanisms were found to account for the observed effects: the primary (electrostatic) salt effect, and the salt influence on the solubility of substrates. In addition, an inhibitory effect of some 2 : 1 and 1 : 2 electrolytes (Na_2SO_4, $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$) appeared at high concentrations. The results suggest that substrates in acetylcholinesterase-catalyzed reactions are extracted into the enzyme microphase at the stage of ES-complex formation and further substrate transformation proceeds inside the enzyme.

INTRODUCTION

The influence of salts on acetylcholinesterase-catalyzed hydrolysis of cationic substrates has been shown to include the primary kinetic salt effect which arises from the variation of electrostatic interaction between the positively charged substrate and the negatively charged enzyme (*1*). On the other hand, many authors have reported on the activation of acetylcholinesterase by some inorganic salts, the activation being more pronounced when divalent cations were present (*2–5*). The specific influences of cations on the active site conformation, directly or through allosteric mechanisms, have been proposed to account for the observed effects.

However, the influence of salts arising from indirect salt-induced medium effects in the activity coefficients of the reactants [the salting-out and salting-in phenomena (*6*)] in both binding and covalent stages (*7, 8*) of the reaction should be considered as well. The salting effects superimposed on the direct electrostatic interactions may be a reason for the above-mentioned "activation" of acetylcholinesterase by inorganic salts. To evaluate the contributions of both effects in the reaction kinetics, data on the influence of various salts over a wide range of concentrations are required.

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Although the influence of salts on the kinetics of acetylcholinesterase reactions has been studied by a number of authors (*1–5*), practically no data are available at ionic strength above unity where the contributions of electrostatic interaction in kinetic constants become insignificant and the importance of the salting phenomena could be separately evaluated (*9*). In order to obtain the necessary experimental data, we have studied the influence of inorganic salts over the range of ionic strength from near physiological to concentrated on the reactions of acetylcholinesterase with neutral and cationic substrates and *N*-methylcarbamylcholine inhibitor.

The data have been interpreted according to the well-documented three-step reaction scheme for acetylcholinesterase-catalyzed hydrolysis,

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} EA + P_1 \overset{k_3}{\rightarrow} E + P_2, \qquad [1]$$

with the steady-state kinetic parameters $k_{cat} = k_2/(k_2/k_3 + 1)$ and $K_m = K_s/(k_2/k_3 + 1)$ (*10, 11*). The salt dependences of the elementary constants K_s and k_2, in accordance with Brönsted equations $K_s = K_s^0 f_E f_S / f_{ES}$ and $k_2 = k_2^0 f_{ES}/f_\ddagger$, can be written as

$$pK_s = pK_s^0 + [\Delta pK_s(C)]_{el} + \Delta\kappa_b C \qquad [2]$$

$$\log k_2 = \log k_2^0 + \{\Delta \log[k_2(C)]\}_{el} + \Delta\kappa_a C, \qquad [3]$$

where C is salt concentration, $\Delta\kappa_b = \kappa_E + \kappa_S - \kappa_{ES}$ and $\Delta\kappa_a = \kappa_{ES} - \kappa_\ddagger$ are the coefficients characterizing salting effects, and "el" denotes an electrostatic term. The parameter κ has its conventional meaning (*6, 12*) as the salting coefficient of a reactant, $\log f_i = \kappa_i C$.

For $\log (k_2/K_s) = \log k_{II}$ we may then write

$$\log k_{II} = \log k_{II}^0 + \{\Delta \log[k_{II}(C)]\}_{el} + \Delta\kappa C, \qquad [4]$$

where $\Delta\kappa = \Delta\kappa_b + \Delta\kappa_a = \kappa_E + \kappa_S - \kappa_\ddagger$. It should be pointed out that k_{II} is always the ratio of elementary constants k_2 and K_s while separate kinetic parameters for binding and acylation, K_m and k_{cat}, may for a particular substrate be available only in the form of complex constants if k_2 in scheme [1] is not rate-limiting.

EXPERIMENTAL

Acetylcholinesterase from cobra *Naja naja oxiana* venom was used after gel filtration on a Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala) column. The specific activity of the enzyme preparation was 3.3 kat/kg protein (1 kat = 1 mol acetylcholine hydrolyzed s^{-1} at 25°C and pH 7.5). The sample of desiccated venom was obtained from the Tashkent Integrated Zoo Plant (Uzbek SSR). Ace-

[1] Abbreviations used: E, enzyme; S, substrate.

tylcholinesterase stock solutions were made in 0.15 M KCl. The concentration of the enzyme active sites in the solutions was calculated from the initial rate of the enzymatic hydrolysis of acetylcholine (2.3 mM) at 25°C and pH 7.5 in 0.15 M KCl, making use of the molecular activity of the enzyme $a_m = 6.33 \times 10^3$ s^{-1} (*13*).

Acetylcholine iodide from Merck was used after recrystallization from an absolute ether/alcohol mixture. 2-(Methylmorpholinium)ethyl acetate iodide and butyl acetate were obtained from "Reakhim," USSR. Butyl acetate from "Reakhim" was redistilled before use. *N*-Methylcarbamylcholine iodide was the preparation used in (*14*). Inorganic salts NaCl, KCl, CsCl, KNO_3, K_2SO_4, Na_2SO_4, $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, and $MgSO_4$ were purchased from "Reakhim" and were of analytical grade.

The rates of the enzymatic hydrolysis of the substrates in salt solutions were followed titrimetrically (Radiometer Titrigraph TTT2/SBR3/ABU12, Denmark) at 25°C and pH 7.5. A 0.01 M KOH solution was used in titration, which was performed in a closed vessel in order to avoid the absorption of atmospheric CO_2.

The k_{cat} and K_m values were calculated from the v^{-1} versus $[S]^{-1}$ plots according to the Lineweaver–Burk transformation of the Michaelis–Menten equation

$$v = \frac{k_{cat}[E]_0[S]}{K_m + [S]}$$

for the reaction scheme [1].

The second-order rate constants, $k_{II} = k_2/K_s$, were calculated from the values of k_{cat} and K_m or from the pseudo-first-order rate constants $k_{obs} = k_{II}[E]_0$ obtained by the differential method of Rudakov (*15*) directly from the first-order kinetic curves at low substrate concentration when $[S] \ll K_m$.

The decarbamylation rate constant k_3 was obtained as follows: the enzyme was carbamylated to 90–95% in the presence of a large excess of *N*-methylcarbamylcholine, the carbamate inhibitor was removed by gel filtration with Sephadex G-25, and the spontaneous reactivation of carbamyl–enzyme was followed by removal of the aliquots from the reaction mixture at appropriate time intervals for titrimetric enzyme assay at 25°C and pH 7.5 in 0.15 M KCl using 2.3 mM acetylcholine as substrate. The k_3 values were calculated from the slopes of log $(v_\infty - v_t)$ versus t plots, where v_t and v_∞ are the enzyme activities at reactivation time t and after the total reactivation of the enzyme, respectively.

The butyl acetate solubilities were determined by measuring the amount of acetic acid released by acetylcholinesterase-catalyzed hydrolysis of the substrate in 0.05-ml aliquots from its saturated solutions which were prepared by equilibrating 0.04-ml portions of the ester with 5 ml of the appropriate salt solutions at room temperature (about 22°C) with occasional shaking for 24 h. From the solubility data the salting coefficients κ_S of butyl acetate in various electrolyte solutions were calculated as the slopes of straight lines according to the Setchenow equation $\log (s_0/s) = \kappa_S C$ as shown in Fig. 1 (due to the low solubility of butyl acetate its activity coefficient in water is close to unity and the solubility ratio s_0/s is the activity coefficient of butyl acetate, f_S). The obtained κ_S values are presented in Table 1.

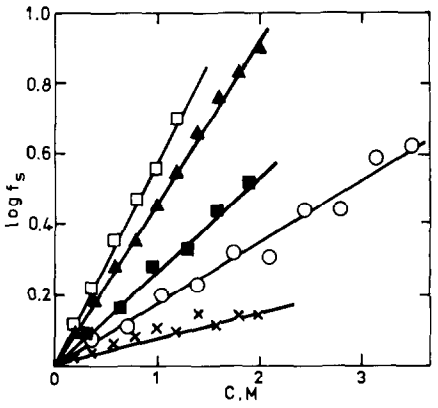


FIG. 1. Influence of salts on the activity coefficients of butyl acetate in water solutions: ×, KNO_3; ○, KCl; ■, $MgCl_2$; ▲, $MgSO_4$; □, Na_2SO_4.

RESULTS

It should be noted first that the preparation of cobra venom acetylcholinesterase was stable in the salt solutions used: no time-dependent inactivation of the enzyme was detected for at least in an hour during incubation. The salt effects observed were not due to changes in the ionization of catalytically active groups in the active site of the enzyme, because pH 7.5, where the measurements were made, remained at all salt concentrations in the limits of the flat pH_{opt} region (*16*).

Neutral substrate

The use of a neutral substrate, *n*-butyl acetate, was suggested by the consider-

TABLE 1

Influence of Salts on Acetylcholinesterase-Catalyzed Hydrolysis of Butyl Acetate

Salt	log k_{II}^{o}	$\Delta\kappa$	κ_S	C_{opt} (M)
KCl	4.510 ± 0.014	0.20 ± 0.08	0.174 ± 0.010	—
CsCl	4.52 ± 0.07	0.15 ± 0.04	—	—
KNO_3	4.487 ± 0.018	0.08 ± 0.02	0.086 ± 0.007	—
K_2SO_4	4.53 ± 0.05	0.60 ± 0.03	—	—
Na_2SO_4	4.52 ± 0.03	0.56 ± 0.06	0.573 ± 0.016	1.5
$MgSO_4$	4.53 ± 0.04	0.45 ± 0.02	0.462 ± 0.007	—
$MgCl_2$	4.51 ± 0.03	0.34 ± 0.07	0.273 ± 0.011	2.1
$CaCl_2$	4.53 ± 0.04	0.25 ± 0.03	—	0.9
$SrCl_2$	4.53 ± 0.05	0.20 ± 0.03	—	0.8
$BaCl_2$	4.51 ± 0.04	0.20 ± 0.07	—	0.4

Note. The parameters of Eq. [4], log k_{II}^{o}, and $\Delta\kappa(\pm SE)$, C_{opt} values of the 1:2 and 2:1 electrolyte effects, and the salting out coefficients $\kappa_S \pm SE$ for butyl acetate.

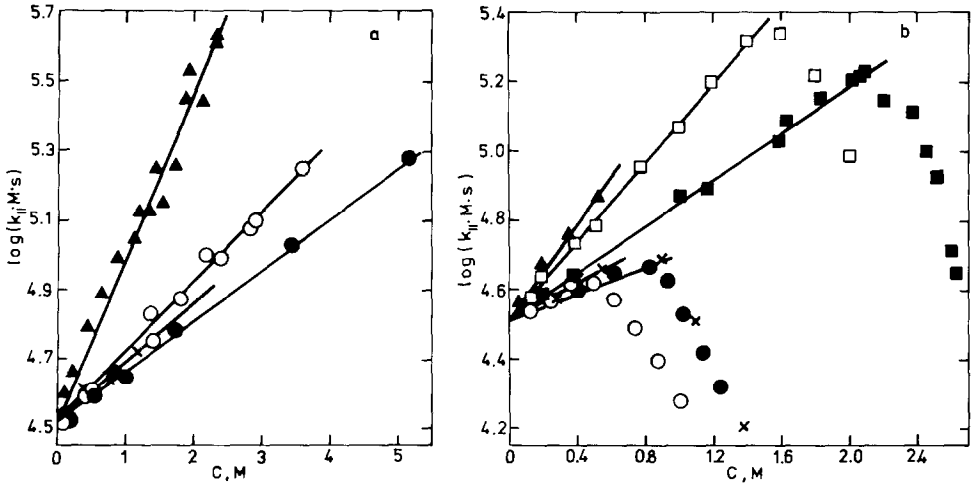


FIG. 2. The second-order rate constant logarithm of acetylcholinesterase-catalyzed hydrolysis of butyl acetate vs salt concentration at 25°C and pH 7.5. (a) ▲, $MgSO_4$; ○, KCl; ×, KNO_3; ●, CsCl; (b) ■, $MgCl_2$; ×, $CaCl_2$; ●, $SrCl_2$; ○, $BaCl_2$; □, Na_2SO_4; ▲, K_2SO_4.

ation that it does not enter into Coulombic interaction with the enzyme and effects other than the influence of salts on the electrostatic interaction can be studied (it has been shown elsewhere (*11*) that the leaving group of butyl acetate is bound at the hydrophobic region in the acetylcholinesterase active site).

In Fig. 2 the plot of the second-order rate constant logarithm of acetylcholinesterase-catalyzed hydrolysis of butyl acetate against salt concentration is given. It is evident from these data that the salts significantly affect the rate constant, and the effect depends on the salt nature. The largest influence which comprises more than 10-fold acceleration is produced by $MgSO_4$. The plots for $MgSO_4$ and all 1 : 1 electrolytes are linear and characterized by a common ordinate intercept (Fig. 2a). Therefore the relationship between log k_{II} and C can be described by Eq. [4] with $\{\Delta \log[k_{II}(C)]\}_{el} = 0$.

At high concentrations of Na_2SO_4 and chlorides of alkaline earth metals an abrupt decrease in enzyme activity is observed (Fig. 2b), so that characteristic optimum concentration values C_{opt} can be assigned to the salts. Still, at suboptimal salt concentrations Eq. [4] is valid for these salts as well, and the common ordinate intercept coincides with that in Fig. 2a. The mean value of k_{II}^{o} for all salts was $(3.31 \pm 0.06) \times 10^4$ M^{-1} s^{-1}. The obtained $\Delta\kappa$ and C_{opt} values are listed in Table 1.

Reference to Table 1 indicates that the $\Delta\kappa$ values for acetylcholinesterase-catalyzed hydrolysis of butyl acetate in various salt solutions and the salting coefficients of the substrate, κ_S, obtained from butyl acetate solubility measurements, are practically identical; statistical treatment of the data according to the equation $\Delta\kappa = \mathbf{a}\kappa_S$ (Fig. 3) has given the slope $\mathbf{a} = 1.012 \pm 0.045$ with the correlation parameters $r = 0.982$ and SD = 0.0418. The results show that the influence of inorganic salts on the enzyme-catalyzed hydrolysis of butyl acetate is

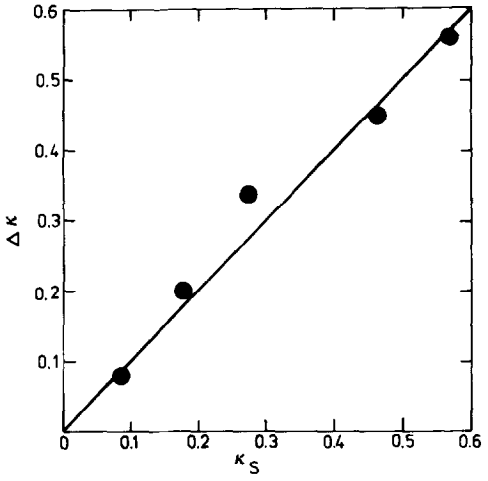


FIG. 3. Correlation between the salting effect parameter for acetylcholinesterase-catalyzed hydrolysis of butyl acetate and the salting-out coefficient of butyl acetate, κ_S. The order of the used salts beginning from the lowest κ_S value is KNO_3, KCl, $MgCl_2$, $MgSO_4$, Na_2SO_4.

determined merely by the influence of the electrolytes on the substrate activity coefficient through the salting-out mechanism.

In order to shed some light on the inhibition phenomenon observed with 1 : 2 and 2 : 1 electrolytes the following experiments were carried out. Acetylcholinesterase was incubated at an above-optimum $MgCl_2$ concentration for 2 h; no progressive loss of enzyme activity was observed. Then the solution was diluted to 1.6 M $MgCl_2$ where the butyl acetate hydrolysis rate corresponded to complete reversibility of the inhibition effect.

In another experiment the enzyme was incubated in 2.6 M $MgCl_2$ for 2 h and centrifuged subsequently at 20,000 *g* for 1 h. Neither spectrophotometric assay at 280 nm nor activity measurements under standard conditions showed any decrease in protein concentration.

An attempt has been made to study the influence of the salts separately on K_m and k_{cat} of the enzymatic hydrolysis of butyl acetate. Since the k_{cat} value 9.11×10^2 s^{-1} for acetylcholinesterase-catalyzed hydrolysis of this substrate is much smaller than the estimated rate constant for the enzyme deacetylation step, $k_3 \cong 10^4$ s^{-1}, acetylation must be rate-limiting in the hydrolysis of butyl acetate, so that k_{cat} gives the value of k_2, and K_m is a good approximation for K_s, the true dissociation constant of the ES complex (*10, 11, 17*). The low solubility of butyl acetate, however, did not allow use of substrate concentrations higher than K_m, which makes the reaction unsuitable for such a study. Nevertheless, the experimental values of K_m and k_{cat} for butyl acetate hydrolysis at 0.15 and 3.0 M KCl presented in Table 2 enable us to suggest that k_2 for this substrate does not depend on KCl concentration and the salt effect concerns only K_s.

Cationic Substrates

For further study of the influence of salts on the elementary kinetic constants of the three-step reaction of substrate hydrolysis by acetylcholinesterase (scheme [1]), we have determined K_m and k_{cat} for two cationic substrates, acetylcholine and 2-(methylmorpholinium)ethylacetate, in the presence of different concentrations of KCl and Na_2SO_4, respectively. The data in Table 2 show that, as observed in the case of butyl acetate, the rate constants k_{cat} do not depend on salt concentration.

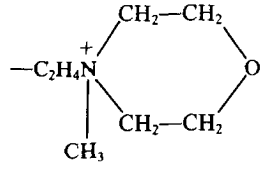
Comparison of the k_{cat} values from Table 2 with $k_3 \cong 10^4\ s^{-1}$ (*11*) implies that, in 2-(methylmorpholinium)ethyl acetate hydrolysis, acetylation is still practically rate-limiting while in acetylcholine the rate constant k_2 must be about 1.5 times larger than k_3, in agreement with earlier observations (*10, 17*) that deacetylation is the rate-limiting step in acetylchline hydrolysis. Thus, a conclusion can be drawn from this analysis that both rate constants, k_2 and k_3, are independent of the concentrations of added inorganic electrolytes.

The conclusion that salts do not affect the acylation and deacylation steps of acetylcholinesterase-catalyzed reactions finds additional support from the data about the influence of KCl on the reaction of acetylcholinesterase with *N*-methylcarbamylcholine which is hydrolyzed by the enzyme through the same kinetic mechanism [1] as are the acetic esters, with the sole difference that the decarbamylation rate constant k_3 is sufficiently low so that K_s, k_2, and k_3 can be determined separately by conventional methods (*18, 19*).

It has been shown elsewhere (*16*) that the rate constant k_2 for carbamylation of acetylcholinesterase by *N*-methylcarbamylcholine does not depend upon KCl

TABLE 2

Influence of Salts on K_m and k_{cat} (±SE) of Acetylcholinesterase-Catalyzed Hydrolysis of Acetic Esters $CH_3C(O)OX$ at 25°C and pH 7.5

X	Salt	C (M)	K (mM)	$k_{cat}\ 10^{-3}$ (s^{-1})
$-C_4H_9-n$	KCl	0.15[a]	17.4 ± 1.6	0.911 ± 0.045
		3.0	4.43 ± 0.70	1.03 ± 0.10
$-\overset{+}{C_2H_4}N(CH_3)_3$	KCl	0.15	0.165 ± 0.013	5.84 ± 0.49
		0.3	0.213 ± 0.016	5.37 ± 0.47
		0.5	0.309 ± 0.024	6.02 ± 0.48
		1.0	0.447 ± 0.036	5.24 ± 0.46
		2.0	0.550 ± 0.043	5.62 ± 0.49
	Na_2SO_4	0.3	2.92 ± 0.14	3.43 ± 0.08
		0.6	3.32 ± 0.15	3.55 ± 0.08
		0.9	2.23 ± 0.16	3.44 ± 0.10
		1.2	2.04 ± 0.08	3.52 ± 0.06

[a] From Ref. (*11*).

TABLE 3

Rate Constants of *N*-Methylcarbamyl–Acetylcholinesterase Decarbamylation (±SE) in the Presence of Different KCl Concentrations at 25°C and pH 7.5

C_{KCl} (M)	k_3 (s^{-1})
0.05	1.92 ± 0.20
0.10	1.93 ± 0.20
0.20	1.93 ± 0.20
0.50	1.93 ± 0.20
0.75	2.00 ± 0.20
1.00	1.95 ± 0.18

concentration. In the present work we have studied the effect of KCl on the *N*-methylcarbamyl–acetylcholinesterase decarbamylation rate constant k_3. The data in Table 3 demonstrate that k_3 is independent of salt concentration.

Since the rate constant k_2 for acetylcholinesterase-catalyzed hydrolysis does not depend upon salt concentration, the salt effect on the second-order rate constant $k_{II} = k_{cat}/K_m = k_2/K_s$ should be identical to the effect on K_s, and in a study of the salt effects in K_s one can use k_{II}. This is demonstrated in Fig. 4 for hydrolysis of acetylcholine. The k_{II} values in Fig. 4 were calculated as the ratios of k_{cat} to K_m from Table 2 or determined independently from the first-order kinetic curves at

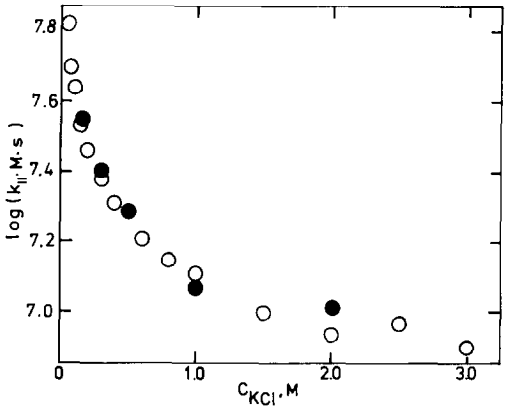


FIG. 4. Influence of KCl on the second-order rate constant logarithm of acetylcholinesterase-catalyzed hydrolysis of acetylcholine at 25°C and pH 7.5: ●, $k_{II} = k_2/K_s$ calculated from the values of k_{cat} and K_m in Table 2; ○, k_{II} values calculated directly from the first-order kinetic curves at low substrate concentration, $[S] \ll K_m$.

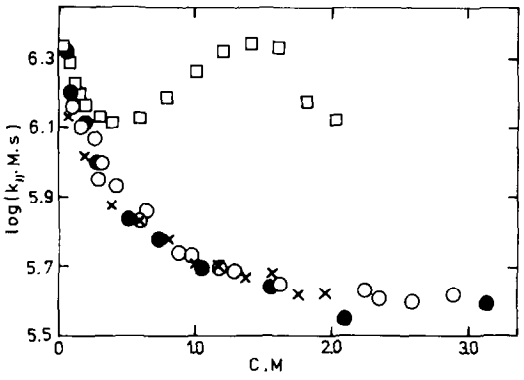


FIG. 5. Influence of salts on the second-order rate constants logarithm of acetylcholinesterase-catalyzed hydrolysis of 2-(methylmorpholinium)ethyl acetate iodide at 25°C and pH 7.5: ●, NaCl; ○, KCl; ×, KNO_3; □, Na_2SO_4.

low substrate concentration when [S] $\ll K_m$. Figure 4 shows that the results obtained by the two methods are consistent. It is important to note that the estimation of k_{II} under the conditions [S] $\ll K_m$ provides smaller errors in comparison with those produced during the determination of k_{cat} and K_m and reduces the amount of experimental work.

Figure 5 shows the influence of different salts upon acetylcholinesterase-catalyzed hydrolysis of 2-(methylmorpholinium)ethyl acetate. The K_m value for this substrate is about 15 times larger than the K_m for acetylcholine; this makes 2-(methylmorpholinium)ethyl acetate a more convenient substrate for studies under the conditions [S] $\ll K_m$ than acetylcholine, which requires reaction volumes not less than 100 ml to get suitable amounts of the product for the pH-stat measurements.

The dependence of log k_{II} upon salt concentration in Figure 5 for KCl, NaCl, and KNO_3 is similar to that for acetylcholine hydrolysis in the presence of KCl in Fig. 4, and qualitatively the relationships represent the electrostatic salt effect curves for the reaction partners of opposite charges. In terms of Eq. [4], $\Delta\kappa$ for these salts is zero. The interpretation of the observed electrostatic salt effect in acetylcholinesterase-catalyzed hydrolysis of 2-(methylmorpholinium)ethyl acetate has been given elsewhere (*20*), as well as experimental data on k_{II} in the range of salt concentrations 50 times lower than those used in the present study.

As can be seen in Fig. 5, for Na_2SO_4 three superimposed salt effects appear in the log k_{II} versus salt concentration plot. The initial decrease in the rate constant with the increasing concentration of Na_2SO_4 from 0.04 to 0.4 M due to the electrostatic effect is followed by an increase in the rate constant due to the salting effect. The salting effect produced by Na_2SO_4 can also be seen in Table 2 as a decrease in K_m as the salt concentration increases from 0.6 to 1.2 M. At concentrations higher than 1.5 M the inhibitory effect of the salt comes in, and an optimum is formed in the log k_{II} versus [Na_2SO_4] plot at 1.5–1.6 M salt, in agreement with the same observation for butyl acetate (Table 1).

DISCUSSION

Since the influence of salts in acetylcholinesterase reactions appears only in the ES-complex dissociation constants ($\Delta\kappa_a = 0$), Eq. [4] for the hydrolysis of butyl acetate can be written as

$$\log k_{II} = \log k_{II}^{\circ} + \Delta\kappa_b C. \quad [5]$$

The equality between the estimated $\Delta\kappa_b$ values and the substrate salting-out coefficients κ_S for the variety of salts (Table 1) implies that $\kappa_E - \kappa_{ES}$ must be zero, or, in terms of activity coefficients, the ratio f_E/f_{ES} in the equation

$$pK_S = pK_S^{\circ} + \log(f_E/f_{ES}) + \log f_S \quad [6]$$

must remain equal to unity at all salt concentrations. This suggests that the expected change in the enzyme molecule upon the binding of substrate is not "visible" to the surroundings.

The persistence of the relation $f_E/f_{ES} = 1$, irrespective of the salt type and concentrations, has been observed earlier in the reactions of chymotrypsin with hippuric acid esters (*7*) and methyl hydrocinnamate (*21*), and it has been interpreted as the support to the concept that the hydrophobic pocket in the active site of chymotrypsin can extract substrates from aqueous medium in a process similar to extraction of a nonelectrolyte from water by an organic solvent (*7*).

Our experiments with acetylcholinesterase and butyl acetate show that the same mechanism may be operative in cholinesterases. The complete absence of salt influence on k_2 and k_3 agrees with the suggestion that substrates are extracted into the enzyme microphase at the stage of ES-complex formation and the further substrate transformation proceeds inside the enzyme. The model agrees with the structure–activity data (*11*) as well as with the EPR spectra of the spin-labeled enzyme (*22*) which show that the catalytically active groups in the acetylcholinesterase active site should be located at the bottom of a jaws-like hydrophobic slit.

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