Acetylcholinesterase: evidence that sodium ion binding at the anionic site causes inhibition of the second-order hydrolysis of acetylcholine and a decrease of its pK_a as well as of deacetylation

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For bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), the Michaelis parameters V_{max} and K_m for the natural substrate acetylcholine were estimated as a function of pH and sodium chloride concentration by the pH-stat method. A single dissociation constant for Na^+ binding ($K = 7 \times 10^{-3} M$) suffices to explain the salt dependence of V_{max}/K_m and of K_m as well as the pH dependence of V_{max}/K_m and V_{max} , K_m being pH independent. This finding provides evidence for a specific effect of Na^+ , presumably by binding at the anionic subsite of the active centre. Na^+ binding causes a 50-fold decrease in k_{cat}/K_m as well as a decrease of one unit in the pK_a of both k_{cat}/K_m and k_{cat} . The intrinsic pK_a in the absence of salt at 25°C is about 7.5. Comparison of the degree of fit of the data to the Debye–Huckel equation, in accordance with an alternative general salt effect, as well as published data for sodium and potassium chlorides also favour a specific salt effect.

Although there are many papers dealing with salt effects on acetylcholinesterase activity, there is no clear understanding of the causes of these effects. It is, however, generally recognized that the rate of acetylcholine hydrolysis at low substrate concentration decreases as the salt concentration increases, while there is a slight increase in activity at high acetylcholine concentration.

It was shown long ago (Wilson *et al.*, 1958) that the pK_a of dephosphorylation, a slow reaction analogous to deacetylation, decreased at increasing salt concentration. Except in the earliest studies, the salt effects were generally interpreted in terms of ionic strength, as in a preliminary report of the present data (Smissaert, 1975). Regrettably, the conclusion in that report about the charge of the catalytic ionizing group could equally well be reversed, as R. M. Krupka convinced me (personal communication).

When it is considered that acetylcholine is a cation itself, it seems worthwhile to re-evaluate the data in terms of a possible specific Na^+ effect and to consider pH dependence as well. For the first time, data are presented that meet the requirements for such an analysis. The picture derived is of surprising simplicity, providing evidence for a specific effect of Na^+ by binding at the anionic site.

The evidence in favour of specific binding is not easily rejectable by limited experiments with sodium

salts of higher anion valency (Nolte *et al.*, 1980) because such anions could have specific effects which would require a check of several assumptions.

Methods

Materials

The acetylcholinesterase used was a partly purified preparation from bovine erythrocytes, obtained from Sigma (type 1, lot 24C-3070). The dry material was dissolved (5 mg/ml) in 0.1 M-NaCl and applied to a Sephadex G-25 column (Sephadex K9/15, bed vol. 9.5 ml), equilibrated and eluted with 0.1 M-NaCl. The eluate, free from other salts, was stored at $-15^\circ C$.

Baker's analysed reagent NaCl, NaOH (Aristar, BDH) and biochemical acetylcholine iodide or acetylcholine bromide (BDH) were used. All other chemicals were reagent grade. Solutions were made in glass-distilled deionized water.

Enzyme assay

Rates of acetylcholine hydrolysis were determined by the pH-stat method with Radiometer equipment (TTTIC with temperature compensation, SBR2C, ABU 12 with 0.25 ml burette, TTA3, G202c glass and calomel electrode).

Reaction vessels contained 50 or 200 ml of reactant, made in boiled water free from CO_2 , kept at 25°C . The pH readings were checked at pH 4.0 and 9.18 as recommended (Albert & Serjeant, 1971). The titrant was a NaOH solution (between 5×10^{-3} and $1 \times 10^{-2} \text{M}$) made in water free from CO_2 . A stream of purified N_2 , equilibrated with the NaCl solution, was applied to the vessels at a rate just sufficient to hold the pH of the enzyme solution in the absence of acetylcholine at the desired value without addition of titrant. Thus the enzyme was always equilibrated at the experimental conditions before the addition of acetylcholine for at least 5 min and no corrections were necessary except for the spontaneous hydrolysis of acetylcholine. Linearity of enzyme concentration against activity was obtained at the four extremes of pH and NaCl concentrations. An enzyme solution preincubated with methylphenylcarbamoyl fluoride (Metzger & Wilson, 1964) had no measurable activity under these conditions, providing evidence that other proteins did not contribute to acetylcholine hydrolysis. The stability of the enzyme over the day was checked by repeating the first experiment at the end. Stability during hydrolysis in the four extreme conditions was checked by keeping the enzyme under these conditions for the time required (about 10 min) before addition of acetylcholine and comparing this value with that obtained without preincubation. Estimations of activity at six pH values at a particular NaCl concentration were always made on the same day.

Estimation of the Michaelis–Menten parameters

All the data were obtained from initial rates. Experiments were carried out with at least three

acetylcholine concentrations, which were about 0.5, 1.0 and 2–3 times K_m , roughly known from preliminary experiments. The third concentration of acetylcholine was selected after calculation of K_m , based on the two rate estimates just made. If the median K_m value obtained was not near the middle of the concentrations used, a fourth concentration was applied.

This limited range was selected because the substrate inhibition constant appeared to be low, relative to K_m , at low NaCl concentrations. With NaCl concentrations lower than 0.2 M, where $K_m < 1.5 \times 10^{-4} \text{M}$, 200 ml reaction vessels were used, and the amount of enzyme and the concentration of the titrant were adapted so as to hydrolyse no more than about 5% of the acetylcholine initially present. Experiments at NaCl concentrations 10^{-3}M and lower were found to give unreliable results, probably because of a slow-time dependent release of protons from proteins in the enzyme preparation. Moreover, pH readings were variable.

Treatment of the data

From the initial rate estimations at three acetylcholine concentrations, three values of $V_{\max}$ and K_m were calculated and their medians determined, for each of the 36 conditions (Table 1). This non-parametric method provides more robust estimates than the usual least-squares methods (Eisenthal & Cornish-Bowden, 1974; Cornish-Bowden & Eisenthal, 1974). The separate K_m medians from experiments at six pH values, always on the same day, were averaged because the day-to-day variation of K_m was found to be greater than that at different pH values at a particular NaCl concentration. The mean K_m of medians of experiments on different days were

Table 1. pH and NaCl dependence of R' and NaCl dependence of K_m

R' is $V_{\max}$ observed relative to the extrapolated pH-independent value. S.E.M. never exceeded 10% of the observed value. Temperature: 25°C . The values in parentheses are the negative slopes of the lines drawn in Fig. 1, between the pH values indicated. These slopes represent $10^{-7} \times K_H$ observed (M^{-1}) and were used to obtain the four best-fitting K_H values (Scheme 3; Table 3). The high $\text{p}K_{\text{a(obs.)}} = 7 + \log$ of the highest values in parentheses.

No. of expts.	NaCl (mM)	pH						High $\text{p}K_{\text{a(obs.)}}$	$10^5 K_m$ (M)
		6.3	6.6	6.9	7.2	7.5	8.0		
4	5	0.22 (0.25)	0.31 (0.38)	0.41 (0.78)	0.54 (1.33)	0.70 (1.35)	0.88	7.13	1.41
4	20	0.25 (0.24)	0.34 (0.49)	0.46 (0.91)	0.61 (1.00)	0.78 (1.00)	0.91	7.0	3.29
3	100	0.35 (0.24)	0.49 (0.43)	0.66 (0.43)	0.78 (0.43)	0.96 (0.43)	0.94	6.63	9.02
3	200	0.40 (0.31)	0.56 (0.31)	0.72 (0.31)	0.84 (0.31)	0.90 (0.31)	1.0	6.49	15.3
2	500	0.51	0.64	0.79	0.85	0.92	0.98	6.34	23.6
2	50	$V_{\max}$ estimates insufficient							5.43

Fig. 1. Plot of k_{cat} relative to the pH-independent k_{cat} estimate ($=R'$, eqn. 3) against $R' \times H$

H , H^+ activity as measured by glass electrode. The polar co-ordinates denote $1/H$, indicated by the pH from 6.3 to 8.0. The filled and open symbols respectively represent R' observed (Table 1) and R' calculated (eqn. 3) with the parameter values of Table 3 ($p = 4$). Concentrations of NaCl (mM): ∇ , 5; Δ , 20; $\square$, 100; $\circ$, 200; $\times$, 500 (only R observed).

used to calculate the standard error of the mean, which was always somewhat less than 10% of the mean. Median V_{max} values at six pH values on one day were plotted against $V_{max} \times H$, where H represents H^+ activity from the pH reading of the experiment. The two highest V_{max} estimates, or more if seemingly fitting a linear relationship (Fig. 1; Table 1), were used to calculate the medians of the intercept with the V_{max} axis and the slope ($-K_{H(obs.)}$).

The pH-independent V_{max} estimates were independent of NaCl concentration. Therefore, all V_{max} values were divided by the median V_{max} intercept, providing k_{cat} relative to the pH-independent value (R' , eqn. 3). By this procedure day-to-day variation due to dilution of the enzyme stock solution and variation in titrant concentration were corrected for without affecting slopes. The R' estimates were averaged (Table 1) and plotted in Fig. 1. Median discrete slopes ($-K_{H(obs.)}$) or those calculated for

each pair of two adjacent pH values, as indicated in Fig. 1, are given in parentheses in Table 1. They were used to obtain best-fitting parameters of eqn. (3) in a non-linear optimization program (Schuhmacher, 1975). Parameter direction search (Hooke & Jeeves, 1961) was used to minimize $\sum wi \cdot (\text{slope}_{obs.} - \text{slope}_{calc.})^2$, where $wi = (1/\text{slope}_{obs.})^2$, the weight required for a constant % error, and 'slope' refers to discrete values, either observed (Table 1) or calculated from eqn. (3).

Results

Initially, experiments were carried out to obtain pH-independent V_{max} estimates as a function of NaCl concentration, as described in the Methods section. The variance of the pH-independent V_{max} estimates with respect to NaCl concentration was much less than that at the same NaCl concentration from day to day. It was therefore concluded that the pH-independent V_{max} estimates were also independent of NaCl concentration. It is thus justified and favourable to divide all V_{max} estimates at six pH values by the pH-independent V_{max} extrapolated as described. The mean relative k_{cat} values (R') obtained in this way are given in Table 1 and Fig. 1. K_m estimates, not affected by the enzyme or titrant concentrations, were found to be independent of the pH between 6.3 and 8.0 at NaCl concentrations between 0.005 and 0.5 M. Means of median K_m are given in Table 1.

Discussion

Minimum conclusions from the data are:

1. At NaCl concentrations between 0.005 and 0.5 M there was no significant effect of pH on K_m . Thus the pH dependence of k_{cat} is very similar to that of k_{cat}/K_m , in agreement with published pK_a values (Krupka, 1966a) in 0.1 M NaCl/0.04 M $MgCl_2$ being 6.32 ± 0.04 and 6.27 ± 0.05 respectively.

2. The pH-independent k_{cat} estimates were also independent of NaCl concentration, and therefore the NaCl dependence of k_{cat} was solely caused by the effect of NaCl on its pH dependence.

3. It follows that the variation of the pH-independent second-order rate constant for acetylcholine hydrolysis (k_{cat}/K_m) depends exclusively on the NaCl dependence of K_m .

In the sections that follow, the effect of different NaCl concentrations on the k_{cat}/K_m estimates at high pH will first be discussed, then the pH dependence of k_{cat} , followed by a comparison of the specific and an ionic strength effect model and then finally a discussion of the k_{cat}/K_m value at high salt concentration.

NaCl dependence of the pH-independent $k_{\text{cat.}}/K_m$ estimates

First, it may be useful to mention that, contrary to the general case, the pH independence of K_m for acetylcholinesterase-catalysed hydrolysis of acetylcholine can certainly not be explained by assuming that K_m equals a pH-independent enzyme-substrate dissociation constant. This is impossible because deacetylation is rate limiting in $k_{\text{cat.}}$ and, moreover, the dissociation constant of acetylcholine should, as does that of other cations, depend on pH (Krupka, 1966b).

The simplest explanation of the fact that $k_{\text{cat.}}$ at high pH is independent of the NaCl concentration whereas $k_{\text{cat.}}/K_m$ at high pH steadily decreases with increasing NaCl concentrations is as follows. When Na^+ adds to the anionic site, the reactivity ($k_{\text{cat.}}/K_m$) of the free enzyme for acetylcholine is reduced, whereas deacetylation is unaffected, as shown by Scheme 1, where E represents the free enzyme and EA an intermediate in the catalytic sequence, probably the acetyl enzyme. Deacetylation should be rate limiting in $k_{\text{cat.}}$ (Krupka, 1964), as will be discussed below.

It can easily be shown that, at high pH, eqns. (1) and (2) apply, where K_{m0} is K_m in the absence of inorganic ions and K_m equals the free substrate concentration, where $v = V_{\text{max.}}/2$, assuming Michaelis-Menten kinetics, α is $k_{\text{cat.}}/K_m$ at high pH and infinitely high NaCl concentration relative to that in the absence of NaCl, and K is an association constant, presumably for addition of Na^+ at the anionic site of the active centre.

$$k_{\text{cat.}}/K_m \text{ (at high pH)} = \frac{k_{\text{cat.}}(1 + \alpha K \cdot [\text{NaCl}])}{K_{m0}(1 + K \cdot [\text{NaCl}])} \quad (1)$$

Because $k_{\text{cat.}}$ is independent of the NaCl concentration:

$$K_m = \frac{K_{m0}(1 + K \cdot [\text{NaCl}])}{1 + \alpha K \cdot [\text{NaCl}]} \quad (2)$$

In this section the NaCl effect will be discussed mainly in terms of K_m , since there is no pH effect on

Scheme 1. Ach, Acetylcholine.

Table 2. Comparison of models based on a specific effect (eqn. 2) and an ionic strength effect (eqn. 4)

Residual relative errors to the data (K_m in Table 1, and best-fitting parameter values (see the text for explanation). Relative error = $\{\Sigma[(K_{m(\text{calc.})}/K_{m(\text{obs.})}) - 1]^2/\text{degrees of freedom (3)}\}^{1/2}$. (b) The application is tentatively rejected. The dielectric constant was assumed to be 78 throughout.

	a Specific effect (Scheme 1; eqn. 2)	b Ionic strength effect (eqn. 4)
Relative error	0.08	0.13
K_{m0} (μM)	8	7
K_{Na} (M^{-1})	148	Z_e -6.0
α	0.022	a (nm) 0.76
		pK_a
		Z_e -2.1*

* Calculated from the pK_a values at the three lower $[\text{NaCl}]$ (Table 1) fitting a linear relationship of pK_a against $\sqrt{I}$.

K_m at all and since it allows easy comparison with data from the literature. Basically, however, the pH-independent reactivity of the free enzyme or the rate of hydrolysis at infinitely low acetylcholine concentration, and thus $k_{\text{cat.}}/K_m$, is involved. It is unsafe to equate K_m in terms of individual rate constants and to discriminate between NaCl effects on affinity and reactivity of the free enzyme for acetylcholine, at least at high salt concentrations.

Whether the data are consistent with Scheme 1 will now be discussed. A non-linear least-squares fit of the mean K_m in Table 1 to eqn. (2) provided the parameter estimates given in Table 2(a). All data were given equal relative weightings because the standard deviation of K_m was found to be rather constant relative to its mean. Thus $\Sigma[(K_{m(\text{calc.})}/K_{m(\text{obs.})}) - 1]^2$ was minimized. The Na^+ association constant $K = 148 \text{ M}^{-1}$ agrees well with the association of NaCl in a dioxan/water mixture with a dielectric constant of about 20 (Fuoss & Hsai, 1967), a reasonable value for a protein surface (Tredgold & Hole, 1976). The best-fitting value of K_{m0} , $8 \mu\text{M}$, provides an estimate of the limiting value of $k_{\text{cat.}}/K_m$ at high pH and in the absence of inorganic salts. Assuming a $k_{\text{cat.}}$ value of about 10^4 s^{-1} for the bovine erythrocyte acetylcholinesterase at 25°C (Schnitzlerling & Nolan, 1975), $k_{\text{cat.}}/K_{m0} \approx 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$. For easy comparison with the data from the literature, $k_{\text{cat.}}/K_m$ at 0.1 M NaCl (Table 1) is about $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, close to that reported for the eel enzyme (Wilson & Harrison, 1961; Rosenberry, 1975a).

The question arises whether this $k_{\text{cat.}}/K_m$ value is high enough to conclude that it largely represents k_{+1} in Scheme 2, as proposed by Rosenberry

Scheme 2.

(1975a,b). Alternatively, $k_{\text{cat.}}/K_m$ may practically equal $k_{+1} \cdot k_{+2}/k_{-1}$, that is to say equilibrium exists between the initial enzyme-acetylcholine complex and the free reactants, or k_{-1} and k_{+2} could, of course, be similar in magnitude.

The question is crucial not only for the interpretation of $k_{\text{cat.}}/K_m$, because if $k_{-1} \approx k_{+2}$, pH and salt effects on the $k_{\text{cat.}}/K_m$ cannot be attributed to those on the free enzyme (e.g. Rosenberry, 1975a).

The answer depends on the k_{+2}/k_{-1} ratio (Scheme 2) (Brocklehurst & Dixon, 1977). For equilibrium k_{-1} should be, for example, 10 times k_{+2} and then $k_{\text{cat.}}/K_m = k_{+1}/[(1 + k_{-1})/k_{+2}] = k_{+1}/(1 + 10)$. Thus $k_{+1} = 11 \times k_{\text{cat.}}/K_m$, e.g. about $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ at high pH and in the absence of inorganic salts, or $k_{+1} \approx 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ in the presence of 0.1 M-NaCl. There is no theoretical reason to reject these values as impossibly high for a diffusion-limited second-order rate constant of acetylcholine with an anionic site at the surface of a relatively immobile protein molecule (Eigen & Hammes, 1963; Kuo-Chen *et al.*, 1975). The value of k_{+2} does not provide any way of rejecting either possibility. The k_{+2} value of the bovine erythrocyte enzyme is not known but must be higher than k_{+3} (Krupka, 1964). If we assume a safe limit for k_{+2} of 10^5 s^{-1} , k_{-1} would be 10^6 s^{-1} . Then the dissociation constant (k_{-1}/k_{+1}) would be about 10^{-4} M at high pH and in the absence of salts. Again there is no evidence to reject this value.

Strong evidence that k_{+2} should be rate limiting in $k_{\text{cat.}}/K_m$, at least with acetylcholine, is provided by the report (Hillman & Mautner, 1970) that the values of $k_{\text{cat.}}/K_m$ for acetylcholine and its sulphur and selenium analogues significantly increase in this same order as do the acidities of the leaving groups. Also relevant in considering the significance of $k_{\text{cat.}}/K_m$ is the estimate by Rosenberry & Neumann (1977) of the bimolecular association rate constant of eel acetylcholinesterase with *N*-methylacridinium to be $1.2 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 0.1 M ionic strength, pH 7.0 and 23°C. The value at high pH certainly exceeds that calculated above as a necessary minimum value for acetylcholine in 0.1 M-NaCl, whereas k_{+1} of acetylcholine should be somewhat higher than that of the much bulkier *N*-methylacridinium molecule. Therefore it can be accepted that $k_{-1} \geq 10 \times k_{+2}$ (Scheme 2), so there is near equilibrium between the enzyme-acetylcholine complex and free enzyme plus acetylcholine, and it is probable that at low salt concentration $k_{\text{cat.}}/K_{m0} \approx k_{+2}/K_{s0}$ and $K_m \approx K_{s0} \times k_{+3}/(k_{+2} + k_{+3})$, as is usually assumed.

Scheme 3.

pH and NaCl dependence of $k_{\text{cat.}}$

Fig. 1 is an Eadie plot of R' against $R' \times H$, of the general form $R' = 1 - R' \cdot H \cdot K_{\text{obs.}}$, where R' is the observed $k_{\text{cat.}}$ relative to the pH-independent limiting value, H is the activity of H^+ as measured by the glass electrode and $-K_{\text{obs.}}$ represents the observed slope. It shows three observations, namely the non-linear pH dependence at low salt, the linear pH dependence with 0.2 M-NaCl and the steady decrease of the initial slope, thus the $\text{p}K_a$, with increasing salt concentration. Two more experimental results should be considered in searching for a scheme to explain the data: the NaCl independence of the limiting $k_{\text{cat.}}$ at high pH and the pH independence of K_m , discussed before. Scheme 3 is in accord with these experimental results, as will be discussed first in qualitative terms.

In considering $k_{\text{cat.}}$, E in Scheme 3 represents the acetyl enzyme, since it is assumed that $k_{\text{cat.}}$ is a measure of deacetylation (Krupka, 1964). The present finding about the close similarity of the $\text{p}K_a$ of $k_{\text{cat.}}$ and $k_{\text{cat.}}/K_m$ at all NaCl concentrations used provides additional evidence that deacetylation is rate limiting in $k_{\text{cat.}}$ with acetylcholine. Otherwise the observed K_a of $k_{\text{cat.}}$ should deviate from that of $k_{\text{cat.}}/K_m$ by the factor $k_{+2}/(k_{+2} + k_{+3})$ (Scheme 2). So this factor is ≈ 1 or $k_{+2} \gg k_{+3}$. The species in the left column all deacetylate with the same pH-independent rate constant practically equal to $k_{\text{cat.}}$. All species in the right column of Scheme 3 are totally inactive. The same Scheme applies to the free enzyme, explaining the $k_{\text{cat.}}/K_m$ behaviour. However, only the free enzyme species E and HE are fully active and thus react with, pH- and NaCl-independent, $k_{\text{cat.}}/K_{m0}$ whereas NaE and NaHE react with a relative $k_{\text{cat.}}/K_m$ equal to α as discussed already.

Non-linearity in Fig. 1 is necessarily caused by more than one ionizing amino acid residue affecting the pH dependence of k_{cat} . A minimum scheme to explain the non-linearity at low salt is given by the equilibria between the lower four enzyme species of Scheme 3, with three independent acid association constants K_n . This is the usual scheme for non-linear H^+ inhibition, where the apparent $\text{p}K_a$ decreases with decreasing pH. To include a possible specific effect of Na^+ binding on the pH dependence, a second group of four Na^+ -complexed enzyme species should simply be added, as shown in the upper part of Scheme 3.

There are some equalities in association constants required by the data. First, the pH independence of K_m requires that $K_5 = K_2$. Secondly, the linear H^+ inhibition at 0.2M-NaCl requires that $K_6 \approx K_4$ and thus $K_7 \approx K_2$. The usual alternative to explain non-linear H^+ inhibition is acid catalysis by an independently ionizing site (K_2). This alternative does not apply here, because Na^+ complexing would not affect the linearity or non-linearity in such a simple scheme.

Eqn. (3) is a steady-state equation for R' written in the form of the Eadie plot used in Fig. 1, including the necessary equalities in association constants discussed above.

$$R' = 1 - R' \cdot H \cdot \left[\frac{\{K_1 + K_2 \cdot K_3 \cdot H + K_4 \cdot K_{\text{Na}} \cdot [\text{NaCl}] \cdot (1 + K_2 \cdot H)\}}{(1 + K_2 \cdot H) \cdot (1 + K_{\text{Na}} \cdot \text{NaCl})} \right] \quad (3)$$

A quantitative check of the fit of Scheme 3 was made by non-linear optimization of the four parameters of eqn. (3) to the data of Table 1. Because estimates of R' are systematically affected by errors in the extrapolated pH-independent k_{cat} ($R' = 1$), the observed slope values were used for optimization. As is clear from Fig. 1, the theoretical R' for the lower salt concentrations differs systematically from the experimental values. However, the mean error of R' for a particular NaCl concentration never exceeded 7%. This error, of course, relates to the extrapolated pH-independent k_{cat} estimate and brings confidence in the procedure used for calculating R' .

A crucial point is whether K_{Na} in eqn. (3) agrees with that obtained from the NaCl dependence of K_m , although it is, of course, possible that affinities differ for the free and acetyl enzyme. Alternatively an optimization procedure with K_{Na} as an additional free parameter should provide a significantly better fit of the slopes calculated to those observed, for instance for a secondary salt effect. So the results of optimization with K_{Na} as an additional free parameter is also given (Table 3). The change of K_{Na} from 148 M^{-1} to 63.3 M^{-1} (Table 3) is probably due to the relative insensitivity of R' to its value and to

Table 3. Best-fitting values of the parameters of Scheme 3 for the pH dependence of k_{cat} .

Values were obtained by non-linear optimization of discrete slope values (calculated) (eqn. 3) to those given in Table 1. Relative error = $\{\Sigma(\text{calculated slope/observed slope} - 1)^2 / (20 - p)\}^{1/2}$, where p = number of parameters (4 or 5, see the text for explanation) and 20 = number of slope values.

K_n^* (M^{-1})	$p = 5$	$p = 4$	$\text{p}K_a$
$10^{-7} K_1$	2.09	3.07	7.49
$10^{-7} K_2$	0.36	0.42	6.62
$10^{-7} K_3$	0.25	0.26	6.42
$10^{-7} K_4$	0.26	0.29	6.46
K_{Na}	63.3	148 (fixed)	
Relative error	0.17	0.18	

* Association constants (Scheme 3).

the high degree of correlation with the K_H values, as is obvious from eqn. (3). However, the increase of free adjustable parameters from four to five does not significantly improve the fit. A lack-of-fit test for experimental and theoretical slopes is not possible because independent variance estimates are not available. It is possible to test mean R' (Table 1)

against those calculated by eqn. (3) with $K_{\text{Na}} = 148$. The within-sample variance of R' relative to its mean is 0.0245, with 60 degrees of freedom (d.f.) (total number of experiments - number of means) and the lack of fit variance is 0.0233 with 20 d.f. (number of means - number of parameters). The variance ratio $F(20/60)$ is thus 0.95, and therefore there is no statistical reason for rejecting the model (Scheme 3). However, the further decrease of the $\text{p}K_a$ of k_{cat} , as well as that of k_{cat}/K_m with an increase of the NaCl concentration from 0.2 to 0.5M is not explained. Presumably secondary interactions are involved, not included in Scheme 3.

The minimum conclusion is that a single site neutralization by Na^+ suffices to explain the varying pH dependence of R' between 0.005 and 0.2M-NaCl. A second conclusion is that the data do not provide any evidence against identity of the Na^+ site as derived from k_{cat}/K_m and k_{cat} , which represent the free enzyme and the acetyl enzyme respectively. It is therefore highly probable that the site that binds Na^+ , effecting a reduction in reactivity of the free enzyme for acetylcholine to 2% ($=\alpha$) and a decrease in $\text{p}K_a$ of a catalytic group in the free enzyme as well as in the acetyl enzyme, without any effect on deacetylation, is the anionic site in the active centre!

The effects of binding of Na^+ to the anionic site are similar to those reported by Krupka (1966b) with tetraethylammonium with acetylcholinesterase from the same source. Tetraethylammonium binds to the free enzyme, preventing second-order hydrolysis of acetylcholine, and also the acetyl enzyme, reducing the deacylation rate by about 20%. Binding of tetraethylammonium as a function of pH showed a $\text{p}K_a \approx 6.3$, similar to that of $k_{\text{cat.}}/K_m$ and $k_{\text{cat.}}$ for acetylcholine hydrolysis. Moreover, binding of tetraethylammonium to the acetyl enzyme prevents protonation of a catalytic group, as neatly demonstrated by an increase in $V_{\text{max.}}$ in the presence of tetraethylammonium at low pH (Krupka, 1966b). Krupka reasonably concluded that there were charge interactions between a cation at the anionic site and a nearby catalytic group, which became inactivated when protonated and apparently neutralized the anionic site.

However, the fit of Scheme 3, with $K_6 \approx K_4$ while $K_1 \approx 10 \times K_3$ (Table 3), shows that addition of Na^+ disrupts or prevents the interaction between the ionizing groups, denoted by K_1 and K_2 , occurring at low salt concentration, and strongly suggests a conformational change on Na^+ binding. A detailed discussion of these interactions is deferred to another paper on the effects of bivalent cations and the nature of the ionizing group denoted by K_1 .

Comparison of the specific effect and general ionic strength effect

Instead of eqn. (1) based on Scheme 1, one could treat the NaCl effect as a primary salt effect on the activity coefficients of the reactants acetylcholine and the anionic site of the active centre by Debye–Huckel theory (Amis & Hinton, 1973). This approach is unpromising as a means of discriminating between the two models because both equations have three parameters. Yet the comparison is made because of the common practice of referring to an ionic strength effect (e.g. Nolte *et al.*, 1980). The equation reads:

$$K_m = K_{m0} \cdot 10 \times \exp. [(A\sqrt{I})/(1 + B\sqrt{I})] \quad (4)$$

According to Debye–Huckel theory, $A = 2 \cdot Z_s \cdot Z_e \cdot \alpha$, where Z_s (the charge of acetylcholine) is 1, Z_e is presumably the charge at the anionic site, and α in water at 25°C assumes the value 0.509; $B = 3.29a$, where a is interpreted as the distance of closest approach and I is ionic strength. Non-linear least-squares optimization was used with equal relative weightings to all K_m estimates, as before. The best-fitting parameter values are given in Table 2 (b). The relative error, defined in Table 2, was 0.13, against 0.08 for the specific site model expressed by eqn. (2) (Table 2). Use of $\log K_m$ to minimize relative error causes it to increase to 0.15.

Thus the specific-site model provides a better fit to the experimental data.

The significance of charge number and distance of closest approach (Table 2, b) is difficult to evaluate. The problem seems largely due to α and the constant in B containing the dielectric constant (D), which has been assumed to be 78, that of the bulk medium, water. For instance, by replacing α by $\alpha' \cdot D^{-3/2}$ and solving $78^{1.5}/D^{1.5} = 6$ or 3, one arrives at, respectively, 1 or 2 negative charges instead of 6 for the reasonable values of $D = 24$ or $D = 37$. These calculations merely serve to illustrate one of the most obvious difficulties in application of Debye–Huckel theory to such studies as this and the empirical nature of the parameters obtained (e.g. Nolte *et al.*, 1980). In my opinion, application of the Debye–Huckel equation does not provide further information if there is no independent chemical evidence about the number of negative charges at the anionic subsite of the active centre.

To prove a specific effect of a particular cation, the most convincing way would be to show that the equivalent salt of another cation is *less* effective, because if the anion is changed one should also consider a possible specific effect of the anion. Data from Dawson & Crone (1973), for instance, show that the sulphate ion significantly decreases K_m , since K_m is lower in 0.4 M- K_2SO_4 than with four 1:1 potassium salts at an equal ionic strength (0.12) as well as with 0.08 M-KCl with an equal K^+ concentration. Unpublished experiments showed sodium citrate to be inhibitory at low acetylcholine concentration and were therefore not continued.

Fortunately the data of Dawson & Crone (1973) show that the initial slopes of K_m against the salt concentration differ significantly ($P < 0.05$) for NaCl and for KCl, being 8×10^{-4} and 13.6×10^{-4} respectively, providing strong evidence that at least K^+ exerts a specific effect on K_m . This conclusion is independent of any theory about the cause of the effect.

A detailed comparison of the effects of lithium sodium and potassium chlorides would be appropriate. However, it may appear that the difference between ion–ion interactions due to specific binding and screening is not as absolute as one would like to think (e.g. Manning, 1979).

High $k_{\text{cat.}}/K_m$ value at high salt concentration

Finally, a complex problem has to be discussed: the significance of α in eqn. (1) or the pathway denoted $\alpha \cdot k_{\text{cat.}}/K_{m0}$ in Scheme 1. The actual value $2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ is still surprisingly high and about 20 times higher than that for 3,3-dimethylbutylacetate, the carbon analogue of acetylcholine. The value was calculated with a $V_{\text{max.}}$ relative to that of acetylcholine of 0.55 and $K_m = 4 \times 10^{-3} \text{ M}$ for the C-analogue obtained with the same acetylcholin-

Scheme 4.

esterase (Purdie, 1969) and substituting $K_{m0}/\alpha = 3.6 \times 10^{-4} M$ (Table 2) for K_m of acetylcholine at infinitely high salt concentration. The value is regarded as high because the possibility that $k'_2 \approx k_{+2}$ (Scheme 4) seems negligible. Moreover, if the anionic site is neutralized by Na^+ , the dissociation constant $K'_s = k'_{-1}/k'_{+1}$ for acetylcholine should be very much higher than that of the C-analogue, because there is simply no force to drive acetylcholine out of the water phase into the hydrophobic active centre, as has been quantitatively analysed (Järv *et al.*, 1976; Langel & Järv, 1978).

Two possible solutions to this problem will only be touched on here and will be treated more extensively elsewhere. Possibly the anionic site consists of two or more negative charges and K'_s for acetylcholine may then be low enough to make the high value of $\alpha \cdot k_{cat}/K_{m0}$ understandable. Secondly, there is the reasonable possibility that $k'_{-1} > k'_{-1}$ (Scheme 4). Thus a significant fraction of $E \cdot NaS$, formed on an encounter of acetylcholine with $E \cdot Na$, dissociates to ES and Na^+ , the pathway being that indicated by the arrow. If so,

$$\alpha \cdot k_{cat}/K_{m0} \neq k'_{+2}/K'_s$$

but a more complicated expression, incidentally, like that given by Rosenberry (1975b) for an induced fit complex.

In considering this proposal, it should be remembered that Na^+ are already in equilibrium with the free enzyme before forming complexes with acetylcholine, because they have equilibrated with the acetyl enzyme! Some combination of these two suggestions could also apply.

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