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REVIEW

Kinetic Salting Effect as a Promising Tool in the Investigation of Enzyme Molecule Changes upon Reaction: Deacylation of Acyl-Chymotrypsins

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Salting-out (salting-in) effects in the kinetics of enzymatic reactions are discussed. The importance of careful consideration of these effects in pK_a's and other equilibrium constants is stressed. Particular attention is given to the kinetic salting effect which reflects enzyme molecule changes upon reaction that affect interactions of the molecule with the surrounding medium. In acyl-chymotrypsin deacylation, the kinetic salting effect depends on whether the substrate acyl group is situated in the substrate binding center (the hydrophobic slit) or not. A new effect, the salt-dependent promotion, has been found; it is caused by a change in the kinetic salting effect upon modifier binding: the bound modifier mimics the substrate acyl group in the hydrophobic slit. These results are explained by the hypothesis that the hydrophobic slit is compressed in the transition state. The observed salt effect also depends on pH. This is explained by salt dependence of pK_a's and individual rate constants. In the neutral pH region the kinetic salting effect, as well as the salting effect in the catalytic center deprotonation constant, K_{a1}, must be considered. At high pH's a conformational change occurs: the group with pK_a 8.8 in the free enzyme displays in acyl-chymotrypsins pK_a values above 11.

Investigation of proteins by physical methods has shown that the protein molecule is a flexible and mobile structure and its atoms undergo a wide variety of motions (*1–4*). These motions are supposed to play a significant role in the enzyme catalysis (*2–8*). Assessment of this supposition, however, goes beyond the capabilities of physical methods as the investigation of the reaction mechanism requires the use of kinetic methods.

As a matter of fact, kinetic data sometimes have been explained by conformational changes which should occur in passing to the transition state of the reaction. In the case of chymotrypsin this hypothesis has been used to interpret the influence of pressure on its stereospecificity (*9*), the variation of activation enthalpy and entropy in the deacylation of a series of acyl-chymotrypsins (*10*), the kinetic isotope effect (*11*), and the dependence of the rate constant on substrate structure (*12*); in model calculations of chymotrypsin stereospecificity the same idea has been used (*13–15*). In these works, however, the conclusion about a conforma-

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tional change in passing to the transition state does not follow unambiguously from the kinetic data, rather it represents a daring hypothesis put forward to explain them. As the question of functional consequences of enzyme molecule mobility is of great importance to theoretical enzymology, there is an obvious need for kinetic methods suitable for detecting and studying conformational changes upon reaction.

In organic chemistry, valuable information about reaction mechanisms has been obtained (*16*) from the investigation of the kinetic salting effect that arises from different salting out (salting in) of the ground and transition states. The appearance of this effect shows that changes which occur upon reaction affect the interaction of the reacting species with the surrounding medium. In the case of an enzymatic reaction, one possible source of this effect would be a conformational change in passing to the transition state.

The importance of salting effects in both equilibrium and rate constants of enzymatic reactions has been pointed out by J. L. Webb in 1963 (*17*). However, enzymologists have not paid to these effects the attention they deserve. So, the influence of salts on chymotrypsin reaction rate constants has been extensively studied but no attempts to explain the observed phenomena by the kinetic salting effect can be found. Let us consider briefly the chymotrypsin reaction scheme and then the results these studies have given.

The chymotrypsin-catalyzed hydrolysis of substrates (usually esters or amides) proceeds in three steps:

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

Here, the enzyme E and substrate S form the Michaelis complex ES, then the acyl-enzyme EA is produced in which the substrate acyl group is attached to Ser-195 of the enzyme by an ester linkage, and the alcohol or amine portion of the substrate, P_1, is released. Finally, the hydrolysis or deacylation of EA occurs producing the acid P_2 and regenerating the enzyme. K_s, k_2, and k_3 stand for the dissociation constant of the Michaelis complex and acylation and deacylation rate constants, respectively.

It is generally agreed that certain cations, effective in concentrations less than 0.01 M (Ag^+, Cu^{2+}, Hg^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Ca^{2+}), bind to chymotrypsin and cause specific salt effects (*18–26*). For the influence of other salts, e.g., KCl, many explanations have been proposed: binding of salt ions converts the enzyme into the active form (*19, 27*) or, by causing a conformational change, enhances its activity (*28*); salt ions encrust the network of hydrogen bonds around the enzyme molecule and in this way stabilize the active conformation (*29*); in salt solution, due to the salting effect, a shift in the enzyme conformational equilibrium occurs, and the fraction of the enzyme in a more active conformation increases (*25*). Quantitative treatment of the data either has not confirmed these proposals (*19*) or has not been ventured at all. In addition, there is spectrophotometric and thermodynamic evidence against attempts to explain the influence of salts at their high concentrations by salt-induced conformational changes: a conformational change

does occur (*30–33*) in the salt concentration range 0 to 0.1 M or 0 to 0.5 M (it depends on pH (*30*)); however, further addition of salt (up to 2 M) causes no changes in physical properties of chymotrypsin solutions (*30, 31*) while the kinetic constant continues to increase (*19, 28, 34*).

Some authors (*34, 35*) have attempted to explain the increase in the rate constant in salt solutions as a stabilization of the transition state vs the ground state. According to (*34*), a pair of positive charges appears in "a kinetically significant step," and an increase in the ionic strength cancels the unfavorable electrostatic interaction. This assumption has been criticized (*25*) for its ad hoc nature. In (*35*) it has been concluded from the enhancement of k_2 in salt solutions that the transition state should be more polar than the ground state; on the basis of the Debye-Hückel theory the authors have proposed that a pair of plus and minus charges should appear in the transition state. The use of the Debye-Hückel limiting law in 1 M salt solution is, however, questionable; in addition, according to the derived equation the transition state should carry the charge plus or minus 1, not plus and minus 1.

Parallel to these works, however, it has been reported that acylation (*30, 31, 36, 37*) or deacylation (*28, 35, 37–39*) rate constants in chymotrypsin reaction do not depend on salt concentration. For the most intensively studied reaction of chymotrypsin with *N*-acetyl-L-tyrosine ethyl ester it has been reported that (i) k_{cat} depends on salt concentration while K_m does not (*40*), (ii) k_{cat} is independent while K_m depends on salt concentration (*35*), and (iii) both k_{cat} and K_m depend on salt concentration (*25, 34*).

It can be concluded that the prospects of investigating the chymotrypsin mechanism with the aid of salt effects have remained unrealized; additional confusion has been introduced instead. Regarding the neglect of salting effects both in kinetic and equilibrium constants as the principal reason for this situation, we have undertaken a careful investigation of salt influence on the deacylation of acyl-α-chymotrypsins with chromophoric acyl groups (*41–46*). This reaction was chosen because its kinetics are convenient to measure with high accuracy.

NONSPECIFIC INFLUENCE OF SALTS ON THE KINETICS OF ENZYMATIC REACTIONS

Addition of salts into the reaction medium may cause various kinetic effects. If they are caused by binding of salt ions to the reactant we have a specific salt effect; otherwise, the effect is called nonspecific. The nonspecific salt effect in the rate constant of an elementary reaction or the primary kinetic salt effect results from changes in the activity of solution components that occur upon addition of salt. It is caused by a change in the ability of water to solvate the ground and transition states of the reaction.

The question of the transition state solvation has been analyzed by E. S. Rudakov and V. P. Tretyakov (*47, 48*). Consider the reaction

$$A \rightleftharpoons X^{\ddagger} \rightarrow P, \qquad [2]$$

where A, $X\ddagger$, and P are the ground state, transition state, and products, respectively. In solution the particles are surrounded by solvation shells. The question is what happens to the ground state solvation shell during the 10^{-13} s the reaction takes place. It has been argued (see (*49–53*) and references in (*48*)) that a complete reorganization of the shell for solvating the transition state is impossible in such short time; that is, the transition state, at least in some processes, is characterized by nonequilibrium solvation. E. S. Rudakov and V. P. Tretyakov have shown (*47, 48*) that this conclusion contravenes the principle of microscopic reversibility. The principle requires that the transition state be solvated as if it were a stable particle, and reorganization of the shell must occur beforehand: random motions of molecules lead to the formation of the transition state solvation shell, and only then the reaction becomes possible.

Thus, there is no principal difference between solvation of the ground and transition states. Addition of salts changes the activity of these states. Salt influence on the rate of Reaction [2] is given by the Brønsted equation,

$$k = k^{00} f_A / f_{X^\ddagger}, \qquad [3]$$

where k and k^{00} are the rate constants in the presence of salt and in the ideal solution, respectively, and f_A and $f_{X^\ddagger}$ are the activity coefficients of A and $X^\ddagger$, respectively. k^{00} is approximately equal to the constant in the absence of salt, k^0.

Salt influence on the activity of a charged solute includes electrostatic as well as salting effects. As will be shown below, in deacylation of acyl-chymotrypsins only the latter is seen (in other cases where the interaction of charges with the medium changes during the process, e.g., in complex formation between the enzyme and a charged ligand, the polyelectrolyte nature of the enzyme may play an important role (*54*)). We address the reader interested in electrostatic effects to Ref. (*54*) and omit here, for simplicity, the electrostatic terms. So we consider the acyl-enzyme here as a nonelectrolyte for which only salting effects are important.

Changes in nonelectrolyte activity caused by addition of salts have been extensively studied (*55–57*). If the activity increases with salt concentration, the effect is called salting out, in the opposite case there is salting in. Changes in the nonelectrolyte activity coefficient, f_n, can be represented (*55*) by a power series in molar (or molal) concentrations of the nonelectrolyte and salt, c_n and c_s, respectively,

$$\log f_n = A_{01}c_s + A_{11}c_n c_s + A_{02}c_s^2 + A_{21}c_n^2 c_s + A_{12}c_n c_s^2 + A_{03}c_s^3 + A_{13}c_n c_s^3 + \cdots, \qquad [4]$$

the parameters A_{ij} depend on the nature of salt and nonelectrolyte. The term $A_{01}c_s$ represents the influence of salt on solvent in diluted solution, and further terms represent the influence of the molecules in pairs, in groups of three, etc. (*58*), they become significant at higher concentrations. If c_n is negligible,

$$\log f_n = \kappa c_s + Bc_s^2 + Cc_s^3 + Dc_s^4 + \cdots, \qquad [5]$$

where $\kappa = A_{01}$, $B = A_{02}$, $C = A_{03}$, and $D = A_{04}$. Conversion of Eq. [3] to logarithmic form and insertion of Eq. [5] into it produces

$$\log k = \log k^0 + \Delta\kappa c_s + \Delta B c_s^2 + \Delta C c_s^3 + \Delta D c_s^4 + \cdots, \qquad [6]$$

where $\Delta\kappa = \kappa_A - \kappa_{X^\ddagger}$, $\Delta B = B_A - B_{X^\ddagger}$, etc. Evidently, on the basis of these parameters, we cannot tell whether the ground and transition states are salted in or out; therefore, it seems proper to use the term "kinetic salting effect"; it is described by Eq. [6]. The parameters $\Delta\kappa$, ΔB, etc., can be defined using the first, second, etc., derivatives of $\log k$ with respect to c_s, e.g.,

$$\Delta\kappa = (d \log k/dc_s)_{c_s \to 0}; \qquad [7]$$

i.e., $\Delta\kappa$ is the initial slope of the $\log k$ vs c_s curve. The range of the kinetic salting effect is determined by the difference in salting coefficients of the ground and transition states, salt effects on those parts of the molecule that remain unaltered during the process make no contribution to this effect. Thus, addition of salt allows one to learn whether there is any difference in solvation of the ground and transition states or, in other words (*36*), is the difference in their structure "visible" from the solution.

In the case of nonenzymatic reactions, the transition state salting coefficient can be calculated from the known salting coefficient of the ground state and the kinetic salting effect, and it can be interpreted on the basis of known regularities in the salting effect (polar nonelectrolytes are salted in, and bulky and nonpolar nonelectrolytes are preferably salted out, etc. (*55–57*)). This approach cannot be applied to enzymatic reactions since only first attempts to interpret protein salting coefficients have been made (*59*). It is possible, however, to determine (i) if there is a kinetic salting effect in the reaction (absence of the effect should be shown with several salts because the coefficients of Eq. [6] for one particular salt may occasionally be close to zero) and (ii) if the kinetic salting effects are similar in reactions of various substrates. Thus we learn whether the reaction is visible from solution and whether the reactions of various substrates can be distinguished from one another.

What can be "seen" from solution during an enzymatic reaction? The change in solvation of the reacting molecules that gives rise to the kinetic salting effect may be caused either by events at the reaction center (breaking and making of bonds, this is the cause of the effect in nonenzymatic reactions) or by a conformational change of the enzyme that alters the interaction of some groups with the medium (such conformational changes are supposed (*60*) to cause kinetic pressure effects, there is a certain parallelism in salt and pressure effects (*61*)).

We now address the question of recognition of the kinetic salting effect. The effect becomes significant at medium and high salt concentrations, and the influence of each salt is individual (cf. the uniform influence of all 1 : 1 electrolytes in the case of an ionic strength effect). The salting-out ability of salts diminishes according to their position in a certain order (*55–57*), the so-called Hofmeister series (*62, 63*); in the case of the kinetic salting effect we have differences in salting coefficients, so the order of salts may be either maintained (*64*) or changed (*65*). If the influence of each salt on the rate constant is individual and salts, in addition, make up a Hofmeister series, we most probably have a kinetic salting effect.

The shape of the salting curves merits a special discussion. In a limited salt concentration range linear dependence of log f or, respectively, log k on c_s has been found (*16, 56*); sometimes the linearity is observed throughout an 8 M salt concentration range (*64*). On the other hand, however, upon wide variation of salt and nonelectrolyte concentrations the terms of Eq. [4] containing even the fourth and fifth powers in solute concentrations may become significant (*66, 67*); curved log k vs c_s dependencies have also been observed (*65*).

Let us proceed now from low-molecular solutes and reactants to proteins. The influence of salts on protein activity as revealed by changes in solubility involves two effects: at low salt concentration ionic atmosphere around charged groups of the protein is formed, this leads to an increase in the protein solubility S; at higher salt concentrations the salting-out effect predominates (*63*). For the latter a linear dependence of log S on c_s has often been assumed (*63*). This assumption, however, is suspect or, at least, needs a careful verification for each particular protein, since acetyltetraglycine ethyl ester (*68*) and other nonelectrolyte model compounds (*69, 70*) display nonlinear salting curves already in the salt concentration range from 0 to 2 M. These nonlinearities have been thought to be due to binding of anions by the peptide group (*68*). However, this explanation might be applied only to concave descending curves while other types of curves also occur; it seems that the salting effect itself (Eq. [4]) could explain these nonlinearities.

It should be noted that enzymologists are accustomed to associating salt influence with some interaction between the enzyme and the salt ion. However, seeking explanations for kinetic effects at medium and high salt concentrations, one should first think of nonspecific effects because there is little doubt as to the change in the reactant activity under these conditions (parallel to that, the binding of ions may cause additional kinetic effects, yet this hypothesis needs a special verification).

SECONDARY SALT EFFECTS

Secondary salt effects are caused by changes in the concentrations of the reactive species. Enzymes may occur in several forms and complexes with different reactivities (protonated and deprotonated forms, monomers and oligomers, complexes with modifiers, etc.). Addition of salt may give rise to specific salt effects due to formation of ion–protein complexes with altered reactivity, as well as cause shifts in the equilibria between various enzyme forms, analogously to the shift in the activation equilibrium (Eq. [3]). Thus secondary salt effects may play an even more important role in enzyme kinetics than in nonenzymatic reactions. These effects are of interest because they can be used for governing the enzyme reactivity (through altering the binding constants of substrates, inhibitors, and modifiers, causing changes in conformation and, correspondingly, in stability of the enzyme etc.) and they can be investigated by both kinetic and physical methods, but, however, they make the investigation of the primary kinetic salt effect a complex task. Neglect of secondary salt effects leads to faulty design of the experiment and contradictory data. Let us consider salt influence on the protonation equilibria and the corresponding data for chymotrypsin.

pK_a's of acids depend on salt concentration, and the shape of the pK_a vs c_s curve depends on the type of the acid (*71*). Most important for protein chemistry are the following types: (a) the acid carries the charge +1, and the conjugated base is neutral (His, Lys, Arg, amino terminal group); (b) the acid is neutral, and the conjugated base carries the charge −1 (Asp, Glu, Tyr, Cys, carboxylic terminal group). For simple model acids it has been shown (*71, 72*) (enzymes as polyelectrolytes may exhibit more complicated dependencies) that

$$pK_a = pK_a^{00} + z\sqrt{\mu}/(1 + A\sqrt{\mu}) + Bc_s + Cc_s^2, \qquad [8]$$

where K_a and K_a^{00} are the experimental and thermodynamic dissociation constants, respectively, z is the charge of the base, μ is the ionic strength, and A, B, and C are empirical parameters. In Eq. [8], the $\sqrt{\mu}$-containing term describes the electrostatic effect on the basis of the Debye-Hückel theory, and the following terms allow for the salting effect.

For chymotrypsin charged forms it has been shown that electrostatic effects in the rate constant pH dependence are abolished on increasing the ionic strength (*34, 73*). With the exception of these effects, however, pK_a's of the catalytic groups were deemed to be independent of salt concentration (*25, 34, 35, 74–76*). In most cases this conclusion was based on two pH dependencies measured at 0.1 M and 1 or 2 M NaCl or KCl concentrations. The result of such a comparison is vague since the error of the calculated pK_a-shift value is the sum of the (quite large) errors of the two pK_a's. One finding directly points out the salt-induced pK_a shift in chymotrypsin: the pH dependencies of k_{cat} measured at various KCl concentrations intersect (*77*); this requires that k_{cat} and pK_a change in the same direction upon addition of salt. This intersection partially explains the controversy in the data noted above: it is seen from Fig. 9 in Ref. (*77*) that at pH's below 7 KCl lowers k_{cat}, between 7 and 8 its influence is weak, and above 8 KCl enhances k_{cat}, the effect being most pronounced at pH 10.

The tradition of considering some kinetic parameters independent of salt concentration merits a special note. It may happen that under a set of conditions (salt concentration range, pH, substrate, and salt nature) changes of some parameters are too small to be measured—the experiment accuracy cannot be infinite. It seems, however, that there also is a tendency to make some of the kinetic parameters independent of salt concentration in order to reduce the amount of the required experimental work and so to "simplify" the interpretation of the obtained data. In the consequence the overall picture may become very intricate.

In our study all the kinetic parameters were taken to depend on salt concentration. Salt effects were interpreted on the basis of the reaction scheme; that is, the functions describing the influence of salt were found for each parameter. These data were used for quantitative description of salt influence on the apparent rate constant, if this proved impossible the scheme was amended. (In Ref. (*28*) a different approach was used: it was tacitly assumed that salt influence on an elementary kinetic parameter may depend on pH, in contrast to the parameter value in the absence of salt. This is, however, inconsistent with the interpretation of results in the framework of the transition state theory according to which (*50*) a pair of the ground and transition states defines one reaction mechanism, the free-

energy difference between them determines the rate constant (only one constant per one mechanism), and the difference in their interactions with surroundings determines the kinetic salting effect (only one $\Delta\kappa$ value with one salt per one mechanism).) For better determination of salt influence on each kinetic parameter high salt concentrations were used. The interesting finding that salt influence on the cinnamoyl-chymotrypsin deacylation is much stronger in 0.1 M NaOH than at neutral pH's (*28*) prompted our measurements at strongly alkaline pH values.

KINETIC SALTING EFFECT IN THE ACYL-CHYMOTRYPSIN DEACYLATION RATE CONSTANT k_3

The influence of salts on the deacylation of furylacryloyl-chymotrypsin (*43*) (Fig. 1) and cinnamoyl-chymotrypsin (*42*) is similar. The effect is not great and high salt concentrations are required to produce it. With some salts the dependencies are linear or nearly linear, and hence the effect can hardly be caused by

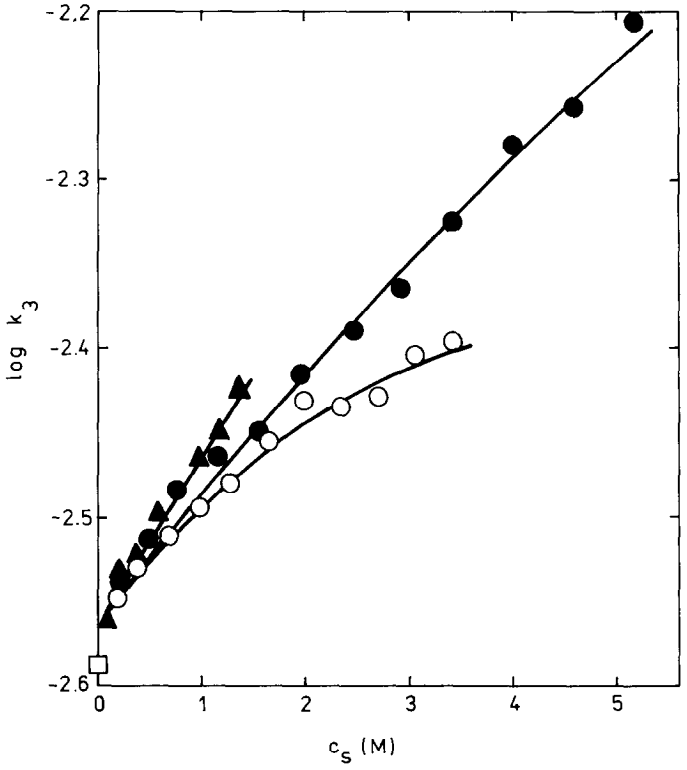


FIG. 1. Kinetic salting effect in the furylacryloyl-chymotrypsin deacylation rate constant k_3 (*43*). Pseudo-first-order kinetics were measured spectrophotometrically at 324 nm, 25.0 ± 0.2°C, pH 9.80, 0.05 M carbonate buffer. The data represent the mean for three to four determinations. Parameter values of the fitted parabolas, Eq. [9], are given in Table 1. (□) Without salt; (▲) Na_2SO_4; (●) NaCl; (○) KCl.

TABLE 1

Kinetic Salting Effect in the Acyl-Chymotrypsin Deacylation Rate Constant k_3

Salt	Parameter of Eq. [9] or [10]	Acyl-chymotrypsin: Cinnamoyl-chymotrypsin (*42*)	Furylacryloyl-chymotrypsin (*43*)	TMAC-CT (*45*)
	$\log k_3^0$	−1.861 ± 0.006	−2.563 ± 0.004	−3.663 ± 0.004
Na_2SO_4	$\Delta\kappa$	0.128 ± 0.025	0.098 ± 0.006	
	ΔB	−0.042 ± 0.020	—	
NaCl	$\Delta\kappa$	0.082 ± 0.010	0.078 ± 0.005	−0.155 ± 0.010
	ΔB	−0.008 ± 0.003	−0.002 ± 0.001	0.036 ± 0.005
	ΔC	—	—	−0.0030 ± 0.0007
KCl	$\Delta\kappa$	0.063 ± 0.009	0.077 ± 0.008	−0.204 ± 0.013
	ΔB	−0.007 ± 0.003	−0.009 ± 0.003	0.047 ± 0.009
	ΔC	—	—	−0.0048 ± 0.0018
CsCl	$\Delta\kappa$	0.033 ± 0.005		−0.363 ± 0.015
	ΔB	−0.005 ± 0.001		0.177 ± 0.014
	ΔC	—		−0.0378 ± 0.0039
	ΔD	—		0.0029 ± 0.0004
	n	38	32	56
	r	0.979	0.994	0.996
	s	0.0112	0.0093	0.0081

Note. Rate constants at pH 9.8 (cinnamoyl- and furylacryloyl-chymotrypsins) or 9.1 (TMAC-CT) have been fitted by least squares to Eq. [9] or [10], respectively. Significance of the terms in the equations has been established by the F test (*78*). Parameter values ± SE are given.

binding of ions. Each salt is exerting an individual effect on k_3, and the influence of the salts weakens according to the Hofmeister series. It can be concluded that the increase in k_3 is caused by the kinetic salting effect. It is described by

$$\log k_3 = \log k_3^0 + \sum_i \Delta\kappa_{k_3,i} c_i + \sum_i \Delta B_{k_3,i} c_i^2, \qquad [9]$$

where c_i is the molar concentration of the ith salt; parameter values are given in Table 1. Similarity of the effects with furylacryloyl- and cinnamoyl-chymotrypsins means that the difference in deacylation patterns of these similar acyl-enzymes is indiscernible from solution.

Comparison of these data with the data about salt influence on the *N*-acetyl-L-tyrosyl-chymotrypsin deacylation (see Fig. 2 for KCl influence; with NaCl and Na_2SO_4 only few data are available (*25*); however, the picture seems to be similar) leads to a less expected conclusion: the last reaction does not differ in the "observable" pattern from the deacylation of cinnamoyl- or furylacryloyl-chymotrypsins; note that the rate constants of these reactions differ by a factor of 10^5. As the kinetic salting effect is insensitive to the interaction of the substrate acyl-amido

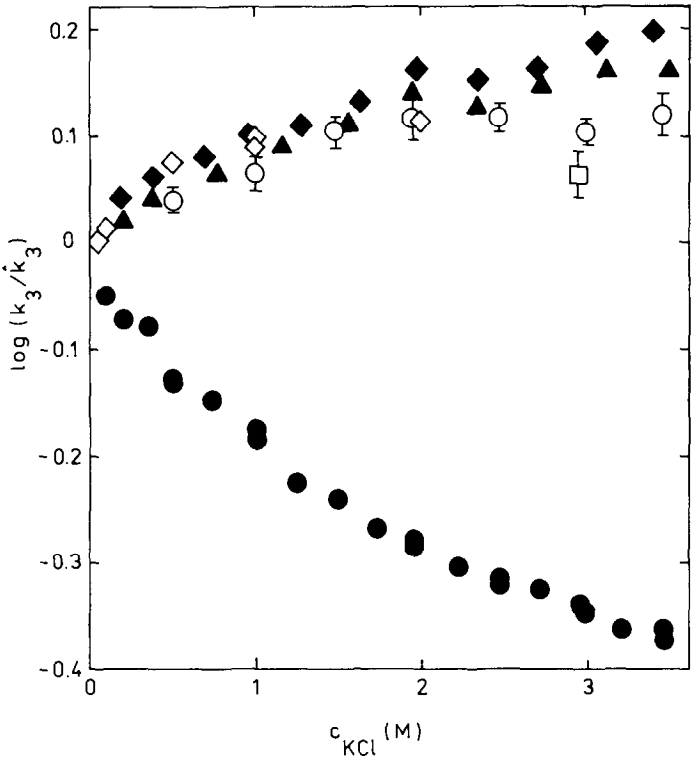


FIG. 2. Dependence of acyl-chymotrypsin deacylation on KCl concentration: (▲) cinnamoyl-chymotrypsin (*42*); (◆) furylacryloyl-chymotrypsin (*43*); (◇) *N*-acetyl-L-tyrosyl-chymotrypsin (the data about KCl influence on k_{cat} in the reaction of α-chymotrypsin with *N*-acetyl-L-tyrosine ethyl ester (*34*); in this reaction $k_{cat} \approx k_3$ (*79, 80*)); (●) TMAC-CT (*45*); (○) the complex of TMAC-CT with indole (*44*); (□) the complex of TMAC-CT with acetonitrile (*44*). The data are normalized, and $\hat{k}_3$ is the rate constant in the absence of KCl (with *N*-acetyl-L-tyrosyl-chymotrypsin in 0.01 M KCl).

group with the corresponding binding site in the chymotrypsin active center, no changes visible from solution should occur in this site upon reaction.

The functioning of the hydrophobic binding center was investigated by the same approach. *p-N,N,N,*-Trimethylammonium-*trans*-cinnamoyl-chymotrypsin (TMAC-CT) was chosen as an acyl-enzyme in which this center should be vacant: in TMAC-CT the bulky and charged *p*-substituent should preclude the binding of the acyl group in the hydrophobic slit; promotion of the TMAC-CT deacylation by indole confirms this (see below).

The influence of salts on the TMAC-CT deacylation is shown in Fig. 3. The picture is entirely different from that seen in Fig. 1, while the difference in the rate constants of these acyl-enzymes is only about 13-fold. The acyl group of TMAC-CT is charged, so one might argue that the effect in Fig. 3 reflects the weakening of electrostatic interactions in salt solution. This possibility, however, can be ruled out: the kinetic salt effect in the deacylation rate constant of the TMAC-CT complex with indole closely resembles that in the cinnamoyl- or furylacroyl-chymotrypsin deacylation (see below) while the introduction of indole into the

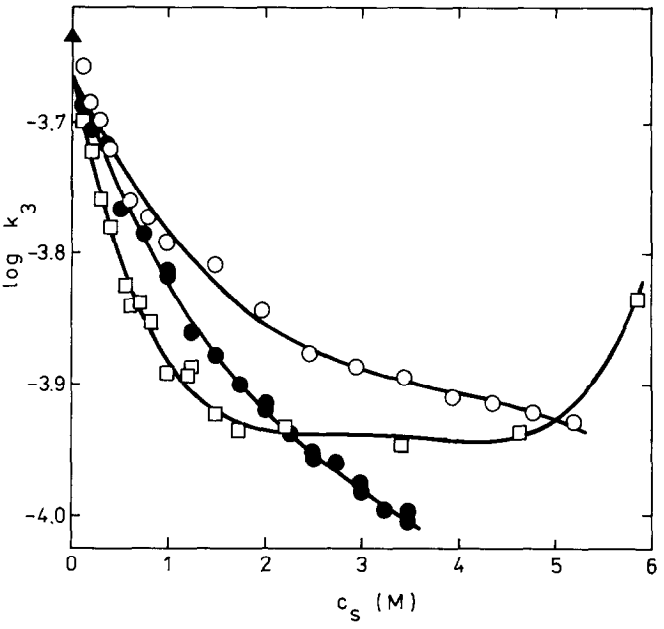


FIG. 3. Kinetic salting effect in the TMAC-CT deacylation rate constant k_3 (*45*). Pseudo-first-order kinetics were measured spectrophotometrically at 299 nm, 25.0 ± 0.2°C, pH 9.1, 0.05 M carbonate buffer. The data represent the mean for three to four determinations. Parameter values of the fitted polynomials, Eq. [10], are given in Table 1. (▲) Without salt; (○) NaCl; (●) KCl; (□) CsCl.

hydrophobic slit should cause no changes in electrostatic interactions between the trimethylammonium group and the charged groups of the enzyme.

The effect in Fig. 3 shows features characteristic of the kinetic salting effect: the influence of each salt is individual, and the order of the salts is the same as those with cinnamoyl- and furylacryloyl-chymotrypsins. The data were fitted to

$$\log k_3 = \log k_3^0 + \sum_{i=1}^{3} \Delta\kappa_{k_3,i} c_i + \sum_{i=1}^{3} \Delta B_{k_3,i} c_i^2 + \sum_{i=1}^{3} \Delta C_{k_3,i} c_i^3 + \Delta D_{k_3,\mathrm{CsCl}} c_{\mathrm{CsCl}}^4. \quad [10]$$

As seen from Table 1, the parameters for TMAC-CT are of opposite sign and much larger than those for the acyl-enzymes considered previously. Absolute values of each parameter of Eq. [10] grow in the order of salts NaCl < KCl < CsCl, and so does the importance of the higher terms: the ratios of the parameter with CsCl to that with NaCl are 2.3 ± 0.3 for $\Delta\kappa$, 5 ± 1 for ΔB, and 13 ± 4 for ΔC. This explains why ΔD is not needed with NaCl. With all of the three acyl-enzymes, the ratio $\Delta B_i/\Delta\kappa_i$ grows in the order of salts NaCl ≤ KCl < CsCl, i.e., in the order of increase in the cation volume. If higher terms in Eq. [4] represent the concerted influence of several salt particles on the characteristics of water (*56*), these tendencies can be expected.

It can be concluded that there are significant differences in the visible pattern of deacylation reactions of TMAC-CT and of those acyl-chymotrypsins in which the

acyl group lies in the hydrophobic slit. These differences should be caused by the interaction of the hydrophobic slit with the ligand. Noncovalent ligands or modifiers were used for further study of this finding.

SALT-DEPENDENT PROMOTION OF THE TMAC-CT DEACYLATION

Deacylation of acyl-chymotrypsins with a vacant hydrophobic slit can be accelerated (promoted) by modifiers. In the corresponding scheme,

$$\begin{array}{ccc} \mathrm{EAH} & \xrightarrow{k_3} & \\ + & & \\ \mathrm{M} & & \\ {\scriptstyle K}\downarrow\uparrow & & \\ \mathrm{EAHM} & \xrightarrow{\alpha k_3} & \end{array} \qquad [11]$$

EAH is the acyl-enzyme form present at slightly alkaline pH values (see below), M is the modifier, EAHM is the modifier–acyl-enzyme complex, K is its dissociation constant, and α is the ratio of the deacylation rate constants of EAHM and EAH. According to Scheme [11],

$$k^{\mathrm{app}} = \frac{k_3 + \alpha k_3[\mathrm{M}]/K}{1 + [\mathrm{M}]/K}, \qquad [12]$$

where the unbound modifier concentration, [M], is given as

$$[\mathrm{M}] = 0.5\left\{[\mathrm{M}]_0 - [\mathrm{EAH}]_0 - K + \sqrt{(K + [\mathrm{EAH}]_0 - [\mathrm{M}]_0)^2 + 4K[\mathrm{M}]_0}\right\}$$

(the subscript zero refers to the total concentrations).

In Fig. 4 the dependence of k^{app} on indole concentration is shown: in the absence of salt indole has no influence on the reaction rate constant, and in KCl solutions the data fit well into Eq. [12]. The results of the data processing by Eq. [12] (in logarithmic form) are given in Table 2. Evidently both K and α depend on salt concentration.

The influence of salts on the binding of indole can be explained on the basis of an equation derived similarly to Eq. [6],

$$\mathrm{p}K = \mathrm{p}K^0 + \Delta\kappa_{\mathrm{p}K}c_{\mathrm{s}} + \Delta B_{\mathrm{p}K}c_{\mathrm{s}}^2 + \cdots, \qquad [13]$$

where $\mathrm{p}K = -\log K$, $\Delta\kappa_{\mathrm{p}K} = \kappa_{\mathrm{EAH}} - \kappa_{\mathrm{EAHM}} + \kappa_{\mathrm{M}}$, $\Delta B_{\mathrm{p}K} = B_{\mathrm{EAH}} - B_{\mathrm{EAHM}} + B_{\mathrm{M}}$, etc. By the least-squares fitting procedure the following equation for description of the data in Table 2 was obtained,

$$\mathrm{p}K = (3.27 \pm 0.03) + (0.15 \pm 0.02)c_{\mathrm{KCl}} + (0.009 \pm 0.002)c_{\mathrm{CsCl}}^2, \qquad [14]$$

$n = 13$, $r = 0.955$, $s = 0.057$; the terms containing c_{KCl}^2 and c_{CsCl} were insignificant according to the F test (*78*).

The K^0 value, 0.53 ± 0.04 mM, is close to the constants of indole binding to the parent chymotrypsin and formyl-chymotrypsin, 0.7 or 0.8 mM (*82, 83*) and 0.46 ± 0.07 mM (*84*), respectively. Thus, TMAC-CT is a well-suited acyl-enzyme for

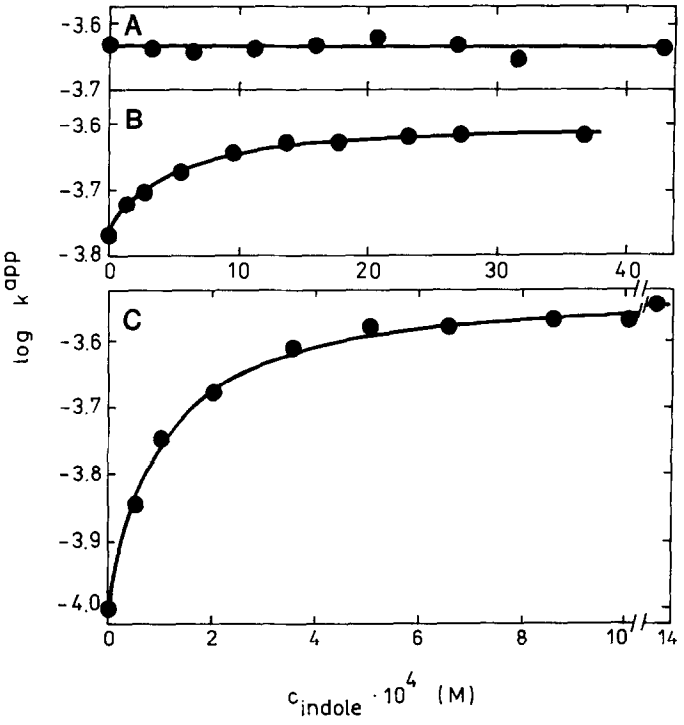


FIG. 4. Dependence of the TMAC-CT deacylation on indole concentration (*44*): (A) in the absence of KCl; (B) in 0.496 M KCl; (C) in 3.46 M KCl. The curves show the fit of the data to Eq. [12] (in logarithmic form). For experimental conditions see Table 2.

TABLE 2

Influence of Salts on the Promotion of the TMAC-CT Deacylation by Indole

c_s	$K \times 10^4$ (M)	α	c_s	$K \times 10^4$ (M)	α
0	No promotion				
		KCl			
0.496	5.3 ± 0.7	1.48 ± 0.02	2.47	2.1 ± 0.2	2.74 ± 0.04
0.991	4.1 ± 0.6	1.76 ± 0.04	2.99	1.8 ± 0.1	2.84 ± 0.04
1.48	3.0 ± 0.3	2.24 ± 0.04	3.46	1.6 ± 0.2	3.10 ± 0.07
1.98	3.3 ± 0.4	2.49 ± 0.06			
		CsCl			
0.601	4.9 ± 0.5	1.78 ± 0.03	3.40	4.0 ± 0.4	2.60 ± 0.06
1.20	4.7 ± 0.4	2.11 ± 0.03	4.63	4.2 ± 0.3	2.71 ± 0.05
2.19	4.4 ± 0.5	2.59 ± 0.06	5.85	2.3 ± 0.4	2.11 ± 0.06

Note. Reference (*44*). Pseudo-first-order kinetics at 25.0 ± 0.2°C were measured spectrophotometrically at 299 nm, pH 9.10, 0.05 M carbonate buffer. The rate constants were determined in duplicate or triplicate at 9 or 10 indole concentrations in each salt solution, and the weighted means of the constants were fitted to Eq. [12] (in logarithmic form; see Fig. 4). Parameter values ± SE, calculated by the nonlinear regression method (*81*), are given.

promotion studies, since its acyl group does not interfere with the binding of modifiers; acetyl-chymotrypsin (*84, 85*) and *N*-formylglycyl-chymotrypsin (*84*), for example, exhibit a much weaker affinity for indole.

Let us consider now the influence of salts on α. It is seen from Fig. 4 and Table 2 that indole promotes the reaction only in the presence of fairly high salt concentrations. This is a new type of promotion—the salt-dependent promotion.

In Figs. 2 and 5, deacylation rate constants of the indole–TMAC-CT complex, αk_3, are shown together with the data concerning salt influence on the deacylation of various types of acyl-enzymes. It is seen that the α value is roughly determined by the distance between the upper and the lower dependencies; figuratively speaking, upon saturation of TMAC-CT with indole the point "rises" from the lower dependency to the upper one.

In order to find out whether the salt-dependent promotion is sensitive to the nature of the modifier, the influence of acetonitrile on the TMAC-CT deacylation was measured under the same set of conditions (Fig. 6). Like indole, acetonitrile does not promote the reaction in the absence of salt (the weak parabolic dependency in Fig. 6A—the change in k_3 is about 20%—is probably a medium effect, similar to the kinetic salting effect). In 2.94 M KCl (Fig. 6B), acetonitrile causes a rate enhancement according to Eq. [12], where $k_3 = (1.06 \pm 0.03) \times 10^{-4}\ s^{-1}$, $K = 0.37 \pm 0.06$ M (cf. the value 0.83 M in the absence of salt (*86*)), and $\alpha = 2.53 \pm 0.09$ ($n = 9$, $r = 0.997$, $s = 0.009$). Due to the side effect (Fig. 6A), the α value may be lowered; nevertheless, it compares well with the corresponding value for indole (Table 2, Fig. 2) while the binding constants of indole and acetonitrile differ by a factor of 2000 (cf. Table 2, similar acceleration of the reaction by modifiers with different binding constants is characteristic of the ordinary promotional effect as well (*87*)).

It can be concluded that the salt-dependent promotion is caused by a change in the kinetic salting effect upon introduction of a modifier into the hydrophobic slit: the pattern characteristic of the TMAC-CT deacylation is replaced by that of acyl-chymotrypsins in which the acyl group is bound in the hydrophobic slit. Evidently, the interaction of the ligand with the slit makes a contribution to the parameters of the kinetic salting effect. If the ligand is a modifier, this contribution appears in the form of the salt-dependent promotion. The contribution seems to be virtually independent of the type of the ligand: it may be acetonitrile, indole, or a covalently bound acyl group as in cinnamoyl-, furylacryloyl-, or *N*-acetyl-L-tyrosyl-chymotrypsin.

CHANGE IN THE KINETIC SALTING EFFECT CAUSED BY THE INTERACTION OF A LIGAND WITH THE HYDROPHOBIC SLIT

Changes in the interaction of the acyl-enzyme molecule with the medium that occur upon formation of the transition state and lead to the appearance of the kinetic salting effect may be caused both by chemical events and by conformational changes. Most likely, the modifier does not change the reaction mechanism: it is bound in the hydrophobic slit at some distance from the reaction center, and

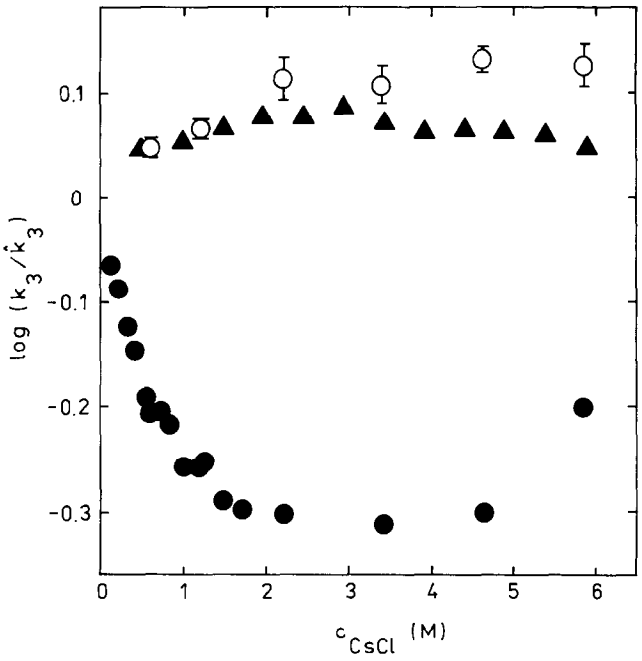


FIG. 5. Dependence of acyl-chymotrypsin deacylation on CsCl concentration: (▲) cinnamoyl-chymotrypsin (*42*); (●) TMAC-CT (*45*); (○) the complex of TMAC-CT with indole (*44*). The data are normalized, and $\dot{k}_3$ is the rate constant in the absence of CsCl.

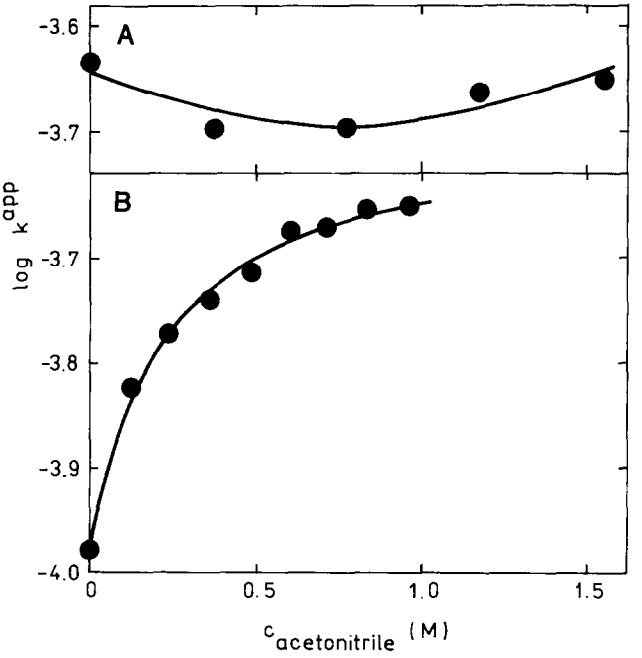


FIG. 6. Dependence of the TMAC-CT deacylation on acetonitrile concentration (*44*): (A) in the absence of KCl; (B) in 2.94 M KCl. (A) A parabola is drawn through the data points (see text). (B) The curve shows the fit of the data to Eq. [12] (in logarithmic form), and parameter values are given in the text. Experimental conditions as in Table 2.

in the absence of salt the binding of a modifier does not affect the rate constant (Fig. 4A). Nor does the modifier shield the reaction center from the medium: it does not just cancel the decelerating influence of salts on the TMAC-CT deacylation; instead, it promotes the reaction to such extent that αk_3 values in salt solutions are higher than $\hat{k}_3$ (Figs. 2 and 5). Hence, the contribution to the kinetic salting effect made by the interaction of the hydrophobic slit with a ligand should reflect some conformational rather than chemical change. Since the contacts of the modifier with the enzyme are confined to the hydrophobic slit, the conformational change should occur in this area (although it may extend to other regions as well).

The question arises as to whether this conformational change occurs only if a ligand is bound in the hydrophobic slit or if it also takes place in the deacylation of TMAC-CT in which the hydrophobic slit is vacant. There is a substantial kinetic salting effect in the TMAC-CT deacylation (Fig. 3, Table 1); hence, major changes should occur in the course of this reaction (it remains unclear, however, what part of the effect is caused by chemical changes at the reaction center). Further, if the conformational change did not occur in the TMAC-CT deacylation, indole and acetonitrile in the hydrophobic slit should be equally able to cause this change. This seems unlikely; in addition to that, such a change in the reaction mechanism should affect the rate constant in the absence of salt as well. The alternative possibility seems more plausible: the conformational change is a characteristic feature of the acyl-chymotrypsin deacylation, but the ligand, whether an acyl group or a modifier molecule, may alter the pattern of the change, visible from solution, and so two types of the kinetic salting effect may occur depending on whether the hydrophobic slit is vacant or occupied.

The ligands under consideration (cinnamoyl, furylacryloyl, and *N*-acetyl-L-tyrosyl groups and indole and acetonitrile) differ from each other in the type of binding (either covalent or equilibrium binding) and in many properties usually considered important to ligand–protein interactions (hydrophobicity, the presence of an aromatic ring, dipole moment, the ability to form hydrogen bonds, etc.). All these properties are irrelevant to the present case since all the ligands are equally capable of making the contribution to the kinetic salting effect. The explanation of the data should be based on a general property common to all the ligands under consideration.

A property that meets this requirement and that can explain the data is the ability of ligands to offer resistance to the compression of the hydrophobic slit: a ligand can most effectively alter the course of such conformational changes in which the enzyme atoms tend to occupy the place of the ligand itself; the ligand is bound in the hydrophobic slit, and so, should compression of this slit occur, all the ligands offer similar resistance to this compression since they are all incompressible and quite similar in thickness (their thickness ranges from 3.5 Å (aromatic rings) to 4 Å (methyl group (*88*)). The role of the ligand can be envisaged as follows: the vacant hydrophobic slit is considerably compressed in the transition state, and concomitantly some changes in the interaction of the enzyme molecule with the medium take place; a similar compression of the hydrophobic slit with a ligand in it is impossible. The changes in the interactions of the enzyme molecule

that occur in passing to the transition state should be different in either cases, this difference gives rise to the contribution to the kinetic salting effect.

The hypothesis that the chymotrypsin hydrophobic slit is compressed in the transition state has already proved useful in explaining the dependence of the constants k_2 and k_3 on substrate hydrophobicity (*12*) and in theoretical calculations of chymotrypsin stereospecificity (*13, 14*). The analysis of the salt effect data has led us to the same conclusion. This hypothesis provides explanations for the different kinetic salting effects in the deacylation of TMAC-CT and of those acyl-chymotrypsins whose acyl groups are bound in the hydrophobic slit, for the salt-dependent promotional effect and for uniform salt-dependent promotion of the TMAC-CT deacylation by indole and acetonitrile.

It may be recalled here that molecules of many enzymes consist of large, extremely compact parts that are separated from each other by clefts; "breathing," i.e., widening and narrowing of these clefts, is one of the normal modes of vibration for such molecules (*89*). This leads to low frequency motions that involve all, or very large portions, of the protein molecule. Motions of that kind have also been found (*90*) in α-chymotrypsin that consists of two domains, and one of them, in its turn, contains a deep cleft—the hydrophobic slit (*91*). In an acyl-enzyme, indoleacryloyl-chymotrypsin, the motions differ from those in the free enzyme (*90*), so they seem to include the breathing of the hydrophobic slit. In view of this theoretical and experimental evidence for the breathing of the hydrophobic slit, the present results may be formulated as follows: functioning of the catalytic center and breathing of the hydrophobic slit are coupled—the reaction does not occur at a random moment of the hydrophobic slit oscillation, it occurs on compression of this slit.

THE DEPENDENCE OF THE INFLUENCE OF SALTS ON pH CONFORMATIONAL CHANGE IN ACYL-CHYMOTRYPSINS IN THE ALKALINE pH REGION

KCl has no influence on the cinnamoyl-chymotrypsin deacylation rate constant at pH 7.2, and at pH 9.8 KCl markedly increases it (*42*). The reason for this is the dependence of cinnamoyl-chymotrypsin pK_{a1} on salt concentration (*42*),

$$pK_{a1} = pK^0_{a1} + \Delta\kappa_{pK_{a1}} c_s, \qquad [15]$$

where $pK^0_{a1} = 7.24 \pm 0.01$ and $\Delta\kappa_{pK_{a1}}$ with KCl is 0.072 ± 0.005 (SE are shown; Eq. [15] is derived analogously to Eq. [6], $\Delta\kappa_{pK_{a1}} = \kappa_{EAH} - \kappa_{EAH_2}$ since salt effect on pH was corrected prior to kinetic measurements). Such a weak dependence of pK_{a1} on c_{KCl} cannot be revealed by comparison of pH sigmoids at 0.1 and 1.0 M KCl concentrations, yet it is sufficient to invalidate kinetic salt effect measurements at pH's below and near the pH optimum.

Investigation of the increased dependence of the cinnamoyl-chymotrypsin deacylation rate constant on salt concentration in strong alkali, first seen by Bender *et al.* (*28*), led to the conclusion that a kinetically significant group deprotonates in the acyl-enzyme at high pH values (*42*). In the corresponding scheme,

$$\begin{array}{ccc} \mathrm{EA} & \xrightarrow{k_3'} & \\ {\scriptstyle K_{a2}}\downarrow\uparrow & & \\ \mathrm{EAH} & \xrightarrow{k_3} & \\ {\scriptstyle K_{a1}}\downarrow\uparrow & & \\ \mathrm{EAH_2} & & \end{array} \qquad [16]$$

EA is the alkaline acyl-enzyme form, and the forms with protonated and deprotonated His-57 are denoted by EAH_2 and EAH, respectively. For the apparent rate constant we obtain the equation

$$k^{app} = \frac{k_3(c_s)\dfrac{a_{H^\cdot}}{K_{a2}(c_s)} + k_3'(c_s)}{1 + \dfrac{a_{H^\cdot}}{K_{a2}(c_s)} + \dfrac{a_{H^\cdot}^2}{K_{a1}(c_s)\cdot K_{a2}(c_s)}}. \qquad [17]$$

According to Eq. [17], salt exerts its influence on k^{app} through k_3, k_3', K_{a1}, and K_{a2}. Salt dependence of k_3 and K_{a1} is described by Eqs. [9] and [15], respectively, and for k_3' and K_{a2} the equations hold

$$\log k_3' = \log k_3'^0 + \Delta\kappa_{k_3'} c_s + \Delta B_{k_3'} c_s^2, \qquad [18]$$

$$pK_{a2} = pK_{a2}^0 + \Delta\kappa_{pK_{a2}} c_s, \qquad [19]$$

where $\Delta\kappa_{pK_{a2}} = \kappa_{EA} - \kappa_{EAH}$. Parameters of Eqs. [18] and [19] (Table 3) will be discussed later. Here the usefulness of salt effect studies should be stressed: they

TABLE 3

Kinetic Parameters for the Description of the Acyl-Chymotrypsin Deacylation in Alkaline KCl Solutions

Parameter of Scheme [16] or [20], Eq. No.	Parameter of the equation	Acyl-enzyme	
		Cinnamoyl-chymotrypsin (*42*)	TMAC-CT (*46*)
k_3', [18]	$\log k_3'^0$	−2.054 ± 0.015	−3.058 ± 0.065
	$\Delta\kappa$	0.396 ± 0.019	0.151 ± 0.044
	ΔB	−0.041 ± 0.005	−0.022 ± 0.009
K_{a2}, [19]	pK_{a2}^0	12.18 ± 0.12	11.34 ± 0.09
	$\Delta\kappa$	0.153 ± 0.042	0.156 ± 0.035
k_{OH}, [22]	$\log k_{OH}^0$		−0.974 ± 0.018
	$\Delta\kappa$		−0.195 ± 0.009
	n	35	66
	r	0.998	0.999
	s	0.012	0.020

Note. Rate constants at various pH values and KCl concentrations have been fitted to Eq. [17] (cinnamoyl-chymotrypsin) or [21] (TMAC-CT) (equations were used in logarithmic form). Parameter values ± SE, calculated by the nonlinear regression method (*81*), are given.

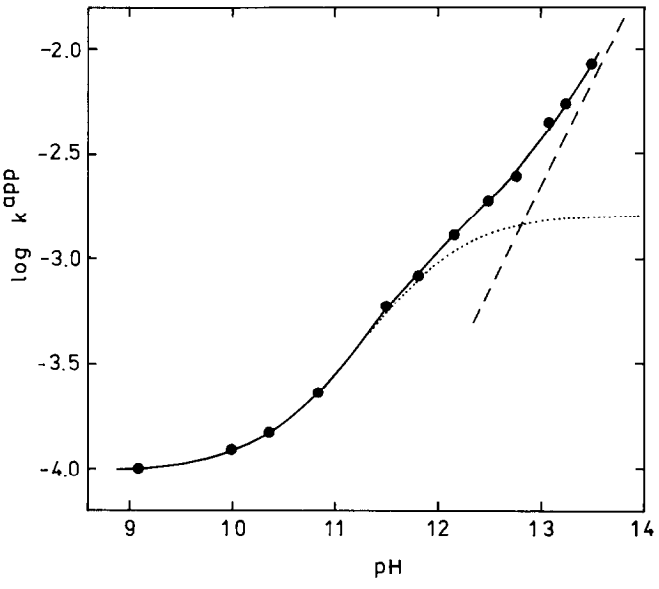


FIG. 7. Dependence of the TMAC-CT deacylation rate constant on pH, 3.4 M KCl (*41*). The data represent the mean for three to four determinations. The curve is calculated with Eq. [21] (in logarithmic form) in which salt-dependent parameters are specified by Eqs. [10] (parameter values in Table 1), [18], [19], and [22] (parameter values in Table 3). By dotted and dashed lines the constituents of the solid curve are shown: a sigmoid (when $k_{OH} = 0$) and a straight line (when $k_3' = k_3 = 0$).

permitted us to demonstrate (*42*) the protonization of a kinetically significant group while in the absence of salt, due to similar k_3^0 and $k_3'^0$ values (cf. Tables 1 and 3), no effect was seen (*28*).

TMAC-CT deacylation in alkaline solutions reveals a new feature (Fig. 7): in the pH range 9.5–12 log k^{app} grows nearly sigmoidally with pH. Then, however, the dependency starts to approximate a straight line with unitary slope. The corresponding scheme (for the high pH range),

$$\begin{array}{ccc} & \xrightarrow{k_3'} & \\ \mathrm{EA} & \xrightarrow{k_{OH}[OH^-]} & \\ K_{a2}\downarrow\uparrow & & \\ \mathrm{EAH} & \xrightarrow{k_3} & \end{array} \qquad [20]$$

involves alkaline hydrolysis of the EA form, in parallel with its enzymatic deacylation. According to Scheme [20],

$$k^{app} = \frac{k_3(c_s)\dfrac{a_{H^\cdot}}{K_{a2}(c_s)} + k_3'(c_s) + k_{OH}(c_s)\dfrac{K_w}{a_{H^\cdot}}}{1 + \dfrac{a_{H^\cdot}}{K_{a2}(c_s)}}, \qquad [21]$$

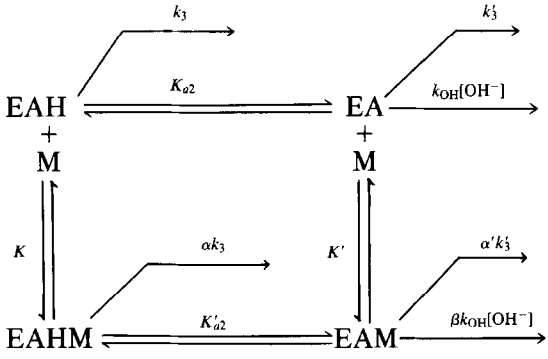
where K_w is the ionic product of water (it is considered independent of c_s since the salt effect on pH was corrected prior to kinetic measurements). Salt dependence of k_3, k_3', and K_{a2} is described by Eqs. [10], [18], and [19], respectively, and for k_{OH} the following equation holds:

$$\log k_{OH} = \log k_{OH}^0 + \Delta\kappa_{k_{OH}} c_s. \qquad [22]$$

In Table 3 the parameters characterizing KCl influence on k_3', K_{a2}, and k_{OH} are given. The k_{OH}^0 value, 0.106 ± 0.005 $M^{-1} \cdot s^{-1}$, may be compared with the alkaline hydrolysis rate constant for *O*-cinnamoyl-*N*-acetyl-serinamide, 0.365 ± 0.005 $M^{-1} \cdot s^{-1}$ (92); thus, alkaline hydrolysis of the TMAC-CT EA form seems to be somewhat hindered in comparison with the model compound. KCl effectively accelerates enzymatic deacylation of the EA forms and decelerates alkaline hydrolysis of TMAC-CT.

It is seen from Table 3 that while these acyl-enzymes reveal quite different pK_{a2}^0 values, the values of $\Delta\kappa_{pK_{a2}}$ coincide. Hence, the difference $\kappa_{EA} - \kappa_{EAH}$ does not depend on the acyl group, and it reflects some changes characteristic of the enzyme molecule itself. The same holds for the difference $\kappa_{EAH^\ddagger} - \kappa_{EA^\ddagger}$: while $\Delta\kappa_{k_3}$ as well as $\Delta\kappa_{k_3'}$ are greatly different, the difference $\Delta\kappa_{k_3'} - \Delta\kappa_{k_3}$ is similar (0.33 ± 0.03 with the first and 0.36 ± 0.06 with the second acyl-enzyme, cf. Tables 1 and 3); as $\Delta\kappa_{k_3'} - \Delta\kappa_{k_3}$ (i.e., $\kappa_{EA} - \kappa_{EAH} + \kappa_{EAH^\ddagger} - \kappa_{EA^\ddagger}$) and $\Delta\kappa_{pK_{a2}}$ (i.e., $\kappa_{EA} - \kappa_{EAH}$) are independent of the acyl group, the difference $\kappa_{EAH^\ddagger} - \kappa_{EA^\ddagger}$ should also be. It was supposed (*41*) that some conformational change should occur in acyl-chymotrypsins at alkaline pH values.

For further investigation of this conformational change indole binding to the TMAC-CT EA form was studied (*46*). On the basis of Schemes [11] and [20] we can write



[23]

where EAM is the complex of the modifier M and the form EA, K' is the dissociation coefficient of EAM, α' and β characterize the change in reactivity upon formation of EAM, and $K_{a2}' = K_{a2}K/K'$. The values of most parameters at a given KCl concentration can be calculated or estimated on the basis of the data in Tables 1 to 3; only K', α', and β are unknown. For determining them, in 3 M KCl at various pH's (from 11.49 to 13.33) the influence of indole on the TMAC-CT deacylation was measured (*46*). Indole caused a 10 to 30% acceleration of the reaction. This indicates the formation of the EAM complex (otherwise the reac-

tion should have decelerated due to pumping of TMAC-CT from EA into EAHM). From the treatment of the data according to Scheme [23] the following values were obtained: $K' = (2.05 \pm 0.31) \times 10^{-4}$ M (for comparison: in 3 M KCl $K = 1.86 \times 10^{-4}$ M), $\alpha' = 1.02 \pm 0.08$ ($\alpha = 2.94$), $\beta = 1.23 \pm 0.03$; $pK'_{a2} = 11.84 \pm 0.07$ ($pK_{a2} = 11.80$). Thus, EA binds indole as effectively as EAH, but there is no salt-dependent promotion with EAM. Since the last effect is connected with the functioning of the hydrophobic slit, the high-pH transition should have some influence on the hydrophobic slit. Differences in the kinetic behavior of TMAC-CT and cinnamoyl-chymotrypsin in this transition (they reveal different pK^0_{a2} values, with cinnamoyl-chymotrypsin $k'_3 \approx k_3$ while with TMAC-CT $k'_3 > k_3$, TMAC-CT in the EA form is subject to alkaline hydrolysis while cinnamoyl-chymotrypsin is not) indicate that functioning of the hydrophobic slit and the high-pH transition are mutually connected. In that the conformational change in acyl-chymotrypsins resembles the transition in the free enzyme characterized by the well-known kinetic pK_{a2} 8.8. The chymotrypsin pK_{a2} tends to shift upward upon modification of Ser-195 (*93*), so it seems reasonable to suppose that the conversion of acyl-chymotrypsins into the EA form corresponds to the conformational change in the free enzyme at pH 8.8.

CONCLUSIONS

It is regrettable that such a fundamental phenomenon as the salting effect in rate constants and pK_a's has hitherto not received the attention it deserves. In many cases this must have led to faulty design of the experiment and/or misinterpretation of the data. The aim of this review was to draw attention to these effects and to show how they could be used for the study of enzyme mechanism. In view of analogy between the salting effect and the influence of organic solvents on the properties of solution (*55, 94*), investigation of these effects in enzyme kinetics should also help one to understand the behavior of enzymes in mixed solvents.

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