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Effect of Sodium Chloride on Substrate Constant and Maximum Velocity in the Enzymic Hydrolysis of *N*-L-Aminoacyl-2-naphthylamines and *N*-L-Aminoacyl-*p*-nitroanilines

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The effect of sodium chloride on the maximum velocity, V, and the substrate constant, K_s, for the hydrolysis of *N*-L-aminoacyl-2-naphthylamines and *N*-L-aminoacyl-*p*-nitroanilines catalyzed by commercial trypsin, chymotrypsin, papain, subtilopeptidase A, leucine aminopeptidase, and purified aminopeptidase B was investigated. The numerical calculations were performed with the aid of a computer programme which applies the method of least squares and calculates the kinetic constants and their standard errors from the Hanes' equation and its extension.

NaCl increased the rate of the reactions catalyzed by aminopeptidase B only (and those catalyzed by trypsin, when the pattern of activation of the enzyme was different from that obtained with aminopeptidase B). In other cases, NaCl either inhibited the enzymes or was without any apparent effect. With aminopeptidase B, the highest rate was measured at approximately physiological (0.154 M) NaCl concentrations. The effect of NaCl was the same on both rat liver and human foetal liver aminopeptidase B.

NaCl at concentrations ranging from 0 to 0.5 M increased K_s in the reactions catalyzed by aminopeptidase B in cases when substrate inhibition was not demonstrable. In leucine aminopeptidase-catalyzed reactions K_s was not affected or it was increased in the presence of increasing amounts of NaCl (tested up to 0.83 M) depending on the substrate.

The results support the earlier assumptions that the activation of aminopeptidase B by physiological chloride concentration is a specific property of the enzyme rather than a consequence of unspecific anion effects.

Anion activation has been termed uncommon for enzymes,[1] and the effects have often been termed unspecific,[2] which in several instances may be true. Recent studies on several enzymes, however, have revealed that anion activa-

tion is not so uncommon, and after the discovery of the classical anion-activation of amylase,[3] several other enzymes have been found to be anion-activated: arylsulphatase,[4] fumarate hydratase,[5] dipeptidyl acylamidase, and cathepsin C,[6,7] *etc.*

Aminopeptidase B (an arginine aminopeptidase which also acts on L-lysyl peptides) has also been found to be activated by physiological sodium chloride concentrations. The effect has been attributed to chloride ions.[8–10] This finding led to studies in these laboratories to elucidate the effects of sodium chloride on the hydrolysis of several *N*-L-aminoacyl-2-naphthylamines and *N*-L-aminoacyl-*p*-nitroanilines catalyzed by leucine aminopeptidase, aminopeptidase B, and several proteinases: trypsin, chymotrypsin, papain, and subtilopeptidase A. An answer to the following question was sought in the study: How does the concentration of sodium chloride affect the maximum velocity V and the substrate constant, K_s, in the reactions mentioned? Several papers on the effects of ions on enzymatic activities have been published in the past, but no paper has concerned the hydrolysis of the above substrates. The task was to find out whether the results obtained could be used to determine the specific anionic activation of aminopeptidase B.

MATERIAL AND METHODS

1. Material. The *N*-L-aminoacyl-2-naphthylamines were obtained from Mann Research Laboratories, New York. The commercial enzyme preparations and other chemicals were the same as published elsewhere.[11] In addition, *N*-L-leucyl- and *N*-L-lysyl-*p*-nitroaniline were purchased from Fluka AG, Buchs, Switzerland. Aminopeptidase B was purified as described earlier.[12] Disc electrophoresis experiments with all the commercial enzyme preparations showed that none of them was pure enough to permit calculation of the molarity of pure enzyme in the reaction mixtures. Hence the rate coefficients could not be calculated.

2. Enzyme assays. All enzyme assays were carried out as described earlier.[11,13,14] It may be added, however, that in assaying the enzymic hydrolysis of *N*-L-aminoacyl-*p*-nitroanilines, the rate of the reaction was measured spectrophotometrically at 410 nm after first stopping the reaction with 30 % acetic acid. Sodium chloride was added to the reaction mixtures [11,14] to the desired concentrations (see Results).

3. Kinetics. The kinetic data were treated numerically as described earlier.[15] The calculations were performed on an IBM 1130 computer and were based on a weighted least squares treatment of the Michaelis-Menten equation in the form proposed by Hanes [16] or of its extension,[15] which takes into account substrate inhibition.

RESULTS

Fig. 1 shows the effect of NaCl on V for the aminopeptidase B-catalyzed hydrolysis of *N*-L-arginyl- and *N*-L-lysyl-2-naphthylamine and *N*-L-lysyl-*p*-nitroaniline. The effect of NaCl was largely the same in all cases. Apparently, only the curve in Fig. 1 E seems to deviate from the general pattern. This may be due to the fact that significant substrate inhibition was found to occur in this case according to the F-test.[15] The extended Hanes' equation leads to considerably greater errors in the derived parameters. The corresponding curve obtained by using the simple Hanes' equation (Fig. 1 E) is in accord with those shown in other cases in Fig. 1. Curves based on both equations are also shown in Fig. 1 D.

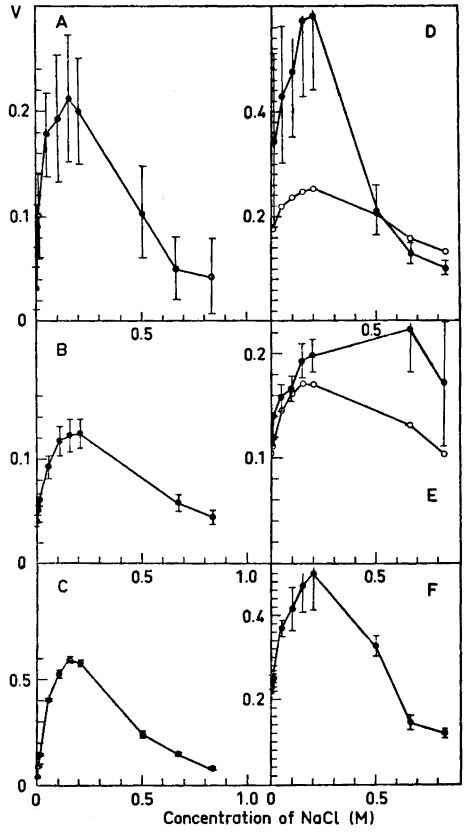

Fig. 1. Effect of sodium chloride on the maximum velocity, V (in $10^{-6} \times$ M min^{-1}) in aminopeptidase B-catalyzed reactions. *A*. Reaction with *N*-L-arginyl-2-naphthylamine catalyzed by rat liver aminopeptidase B. *B*. Reaction with *N*-L-lysyl-2-naphthylamine catalyzed by rat liver aminopeptidase B. *C*. Reaction with *N*-L-lysyl-*p*-nitroaniline catalyzed by rat liver aminopeptidase B. *D*. Reaction with *N*-L-arginyl-2-naphthylamine catalyzed by human foetal liver aminopeptidase B. *E*. Reaction with *N*-L-lysyl-2-naphthylamine catalyzed by human foetal liver aminopeptidase B. *F*. Reaction with *N*-L-lysyl-*p*-nitroaniline catalyzed by human foetal liver aminopeptidase B. The vertical lines represent the standard errors of the points (this applies to all other figures too). In *D* and *E*, the open circles represent points calculated by a first-degree equation. Concerning the computer calculations, see text and Ref. 15 for more details.

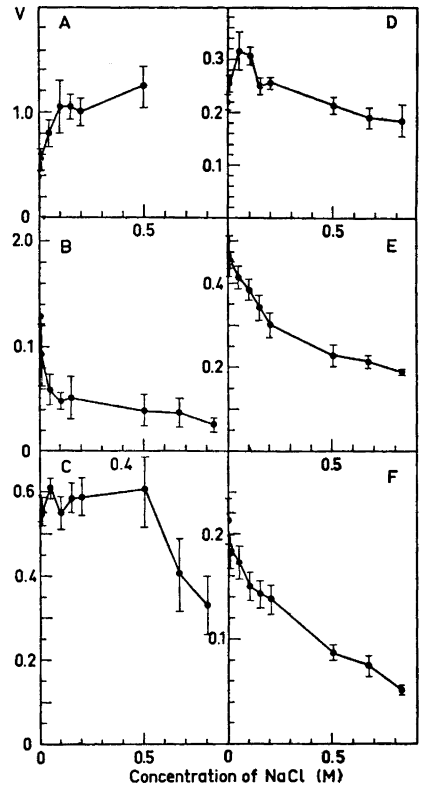

Fig. 2. Effect of sodium chloride on the maximum velocity, V (in $10^{-6} \times$ M min^{-1}), in the enzymic hydrolysis of some *N*-L-aminoacyl-2-naphthylamines. *A*. Reaction with *N*-tosyl-L-arginyl-2-naphthylamine catalyzed by trypsin. *B*. Reaction with *N*-L-leucyl-2-naphthylamine catalyzed by α-chymotrypsin. *C*. Reaction with *N*-L-alanyl-2-naphthylamine catalyzed by leucine aminopeptidase. *D*. Reaction with *N*-L-alanyl-2-naphthylamine catalyzed by papain. *E*. Reaction with *N*-L-methionyl-2-naphthylamine catalyzed by α-chymotrypsin. *F*. Reaction with *N*-L-phenylalanyl-2-naphthylamine catalyzed by leucine aminopeptidase.

Fig. 2 shows results obtained with some other enzymes. In most cases the general pattern revealed inhibition by sodium chloride (except for trypsin). Fig. 3 shows the inhibitory effect of sodium chloride on the maximum velocity, V, in the subtilisin-catalyzed hydrolysis of N-L-leucyl-2-naphthylamine.

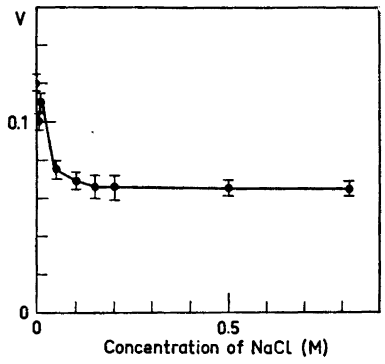

Fig. 3. Effect of sodium chloride on the maximum velocity, V (in $10^{-5} \times$ M min^{-1}), in the subtilisin-catalyzed hydrolysis of N-L-leucyl-p-nitroaniline.

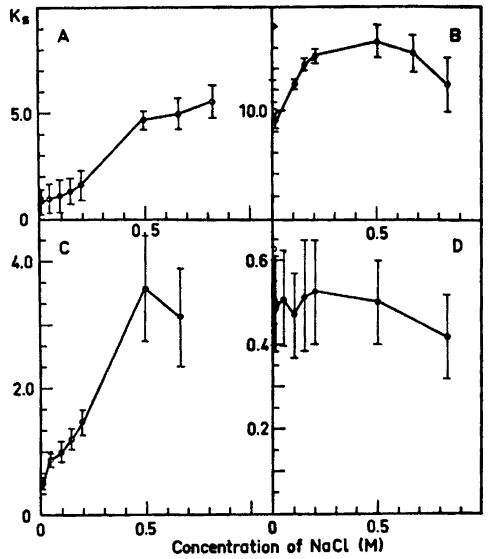

Fig. 4. Effect of sodium chloride on the substrate constant, K_s (in $10^{-4} \times$ M), in some aminopeptidase-catalyzed hydrolyses of N-L-aminoacyl-2-naphthylamines and N-L-aminoacyl-p-nitroanilines. *A.* Reaction with N-L-lysyl-2-naphthylamine catalyzed by human foetal liver aminopeptidase B. *B.* Reaction with N-L-lysyl-p-nitroaniline catalyzed by rat liver aminopeptidase B. *C.* Reaction with N-L-alanyl-2-naphthylamine catalyzed by leucine aminopeptidase. *D.* Reaction with N-L-phenylalanyl-2-naphthylamine catalyzed by leucine aminopeptidase.

The effect of sodium chloride on the substrate constant, K_s, is shown for some cases in Fig. 4 for aminopeptidase-like enzymes, and in Fig. 5 for α-chymotrypsin and papain.

The effect of sodium chloride on v_0 (rate at low substrate concentrations) at several substrate concentrations (ranging from 0.0166 mM to 0.83 mM) was also studied without numerical analysis by plotting the rate attained at certain concentrations of the substrate against the concentration of sodium chloride. The results were of a similar nature as those shown in Figs. 1 – 3. This concerns particularly the trypsin-catalyzed reactions. Sodium chloride inhibited all leucine aminopeptidase-catalyzed reactions. With α-chymotrypsin

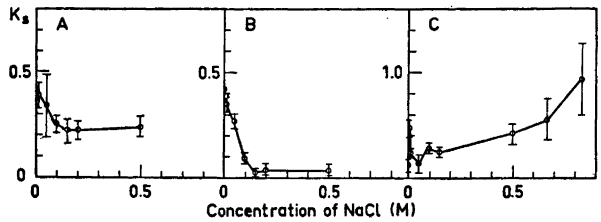

Fig. 5. Effect of sodium chloride on the substrate constant, K_s (in $10^{-4} \times$ M), in reactions catalyzed by two proteinases. *A*. Reaction with *N*-L-leucyl-2-naphthylamine catalyzed by α-chymotrypsin. *B*. Reaction with *N*-L-methionyl-2-naphthylamine catalyzed by α-chymotrypsin. *C*. Reaction with *N*-L-alanyl-2-naphthylamine catalyzed by papain.

it was observed that the higher the substrate concentration, the stronger was the inhibition caused by sodium chloride. At certain low substrate concentrations (0.05 mM), with *N*-L-leucyl-2-naphthylamine and *N*-L-methionyl-2-naphthylamine, a maximum was observed in the v_0 *versus* [NaCl] curves at 0.05 – 0.10 M sodium chloride. Furthermore, it was found that the pH-dependence curves obtained in the absence and presence of sodium chloride (0.2 M) were of approximately the same form in all of the reactions investigated in this study (catalyzed by the aminopeptidases and proteinases, using *N*-L-arginyl-2-naphthylamine for aminopeptidase B, *N*-L-leucyl-2-naphthylamine for leucine aminopeptidase, α-chymotrypsin, and subtilisin, *N*-benzoyl-DL-arginyl-2-naphthylamine and *N*-tosyl-L-arginyl-2-naphthylamine for trypsin and papain). The most noticeable result was strong activation of aminopeptidase B by NaCl. The reactions were performed in 0.025 M phosphate, borate, and β,β-dimethylglutarate buffers.

DISCUSSION

The calculated values of the maximum velocity V at high substrate concentrations are more accurate than those of the substrate constant K_s. Therefore, Figs. 1 – 3 may be said to describe the effect of sodium chloride on V correctly. In several cases (not shown) the errors of K_s made the consideration of the effects of sodium chloride on K_s impossible.

The salt effects seemed to depend on substrate inhibition. This may, however, only be due to the lesser accuracy of the calculated parameters when the extended Hanes' equation was used (see above) and is not real.

Webb [1] has reviewed the effects of salt concentration and ionic strength on enzyme activity. The salt effects revealed in this paper appeared at such low salt concentrations that the contribution of the salts to the solvent may be of minor importance. It could be assumed that sodium chloride affects directly or indirectly the interaction between the enzyme and the other components of the system.

The addition of sodium chloride has been shown to increase the catalytic rate constant and decrease K_m in the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate, both of these effects leading to a faster overall reaction.[17]

With increasing concentration of sodium chloride, a decrease in K_s was observed. When higher concentrations of salt are added, conformational changes in the enzyme protein may occur,[18–20] or the enzyme protein may disaggregate.[21] In some cases, the relative effect of anions is said to be influenced by the nature of the activating cation.[22] This is also possible in reactions catalyzed by aminopeptidase B.[10] It has also been reported that when transaminase binds the substrate, there are no conformational changes, but the substrate simply displaces the masking tris-chloride or sodium chloride ions from a positive enzyme site.[23] With ribonuclease it has been found that there are six sites which bind Cl^- ions,[24] and similar effects can be seen with the synthetic quaternized polymer polyvinylpyridine which may bind an excess of bromide counterions.[25] Numerous other cases of activating or inhibiting salt effects are found in pertinent works on the subject. In the case of aminopeptidase B, efforts to explain the anionic activation have been made.[9,10]

The results of this study showed that in the many enzyme reactions tested, only in those catalyzed by aminopeptidase B did sodium chloride produce maximum activation at physiological concentration (close to 0.15 M). This was the case with all aminopeptidase B substrates and with both rat liver and human foetal liver enzyme. Earlier suggestions as to the role of aminopeptidase B have been proposed on the basis of the salt effects,[10,26] and the results in this paper can be interpreted as meaning that aminopeptidase B could be specifically activated by sodium chloride, whereas the effect of the salt on the other enzymes tested may be of more or less unspecific nature. This is evidenced also by the finding, made in these laboratories with a number of different enzyme preparations, that only the enzymic hydrolysis of *N*-L-arginyl- and *N*-L-lysyl-2-naphthylamine is noticeably accelerated by sodium chloride and other similar salts, although almost thirty other substrates (*N*-L-aminoacyl-2-naphthylamines) have been tested. However, if enzymes other than aminopeptidase B cleave the substrates mentioned, only a faint activation by NaCl is observed. However, *Micrococcus lysodeikticus* polynucleotide phosphorylase, for example, reaches its maximum activity at 0.2 M potassium chloride.[27]

The right part of the *V versus* [NaCl] curves of aminopeptidase B were approximately bell-shaped. Webb [1] states that hardly all such curves can be explained on the basis of ionic strength alone. The nature of the activation of aminopeptidase B by sodium chloride is also elucidated by the form of the rate *versus* pH curves, obtained with and without NaCl. Combination with negative chloride ions would produce a displacement to the right, but the curves most clearly differed in height. (As mentioned in the results, the other enzymes tested produced very similar curves in height and position.) The optimum pH of aminopeptidase B-catalyzed reactions can be displaced, but this occurs at higher inhibiting phosphate concentrations.[10] Sodium chloride affects the substrate constant in all aminopeptidase B-catalyzed reactions. Hence the salt could affect the combination of the enzyme to the substrate as suggested earlier.[9] With aminopeptidase B no disaggregation of the enzyme protein to subunits has been observed in the presence of activating concentrations of sodium chloride.

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