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EFFECT OF BILE ACIDS AND IONIC STRENGTH ON TRYPSINOGEN ACTIVATION BY HUMAN ENTEROPEPTIDASE

H. RINDERKNECHT * and R. FRIEDMAN

Veterans Administration Hospital, Sepulveda, Calif. 91343, and Department of Medicine, School of Medicine, University of California, Los Angeles, Calif. 90024 (U.S.A.)

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Summary

(1) Increasing ionic strength diminishes the rate of enteropeptidase-catalyzed activation of trypsinogen by increasing dissociation of the Michaelis complex and, thus, K_m of the reaction.

(2) The effect of ionic strength depends not only on the magnitude of this parameter, but also on the nature of the anion. Enzyme activity is diminished progressively by isoionic concentrations of ClO_4^-, Cl^- and SO_4^{2-} in this order. The influence of cations of the alkali series is comparatively insignificant.

(3) Bile acids increase the rate of trypsinogen activation by human enteropeptidase (EC 3.4.21.9). Derivatives of cholic acid are less active than those of deoxy- and chenodeoxycholic acid. Triton X-100 is inactive.

(4) The effect of bile acids on enteropeptidase-catalyzed trypsinogen activation is independent of the ionic strength except at very low I values where anomalous kinetics are observed. Bile acids probably decrease dissociation of the Michaelis complex since 2.5 mM glycodeoxycholic acid has no significant effect on K_m, but increases V about 2-fold over a wide range of ionic strengths (0.016—0.073).

(5) Bile acids have no effect on enteropeptidase-catalyzed hydrolysis of the synthetic substrate *N*-benzoylarginine ethyl ester, suggesting that their mode of action may involve an enzyme subsite with specific affinity for the trypsinogen molecule.

Introduction

It was first shown by Kunitz [1] that activation of trypsinogen by low concentrations of enteropeptidase results in the production of considerable

* To whom requests for reprints should be addressed.

amounts of inert protein and low yields of trypsin. Hadorn et al. [2] recently reported that formation of inert protein could be minimized by addition of bile acids to the activation mixture and that 2.5 mM glycodeoxycholic acid caused an approx. 6-fold increase in the velocity of trypsinogen-activation by rat enteropeptidase. Similar results were obtained when human pancreatic juice was used as a source of trypsinogen and small-intestinal homogenates as a source of enteropeptidase. Nordström [3] was able to confirm the effect of bile salts on rat enteropeptidase, but other workers recently failed to obtain any evidence of activation of the human enzyme by bile [4]. Several investigators including ourselves have reported that the activity of enteropeptidase is also greatly affected by the ionic strength of the medium [5—7]. We have, therefore, reinvestigated the bile salt activation of human enteropeptidase and studied the influence of the ionic environment on this system.

Materials and Methods

Enteropeptidase was prepared free of trypsin and other pancreatic enzymes by gel filtration of human duodenal juice as described previously [7]. Trypsinogen, (1× crystallized, salt free) and trypsin, (2× crystallized, salt free) were purchased from Worthington Biochemical Corporation, New Jersey.

Enteropeptidase assay. The reaction mixture contained: 0.25 ml 50 mM Tris-maleate buffer (pH 5.3)/0.6 ml water (or solutions containing varying amounts of NaCl or glycodeoxycholic acid, or both)/0.1 ml enteropeptidase solution ($2.5—13 \cdot 10^{-4}$ mI.U., ref. 7). 0.1 ml of a freshly prepared solution of trypsinogen (26 mg/10 ml 5 mM Tris · HCl, pH 5.5) was then added to start the reaction. The mixture (pH 5.6) was incubated at 25°C for 15 min. Aliquots of 10 µl of the above mixture were removed and added to 0.6 ml 50 mM Tris · HCl buffer (pH 8.5) and 0.4 ml Z-Gly-Gly-Arg-NapNH (80 mg/100 ml water). After incubation for 15 min at 25°C 0.1 ml 1 M citrate buffer (pH 4.5) was added to stop the reaction. Fluorescence intensity was read in an Aminco-Keirs spectrophotofluorometer (excitation at 325 nm, emission at 415 nm, uncorrected) and the trypsin content determined with the aid of a standard graph obtained with bovine trypsin under the same conditions except that the Tris · HCl buffer contained Triton X-100 (1 : 4000). Enteropeptidase activity observed is expressed as mI.U. trypsin generated/ml.

Enteropeptidase-catalyzed hydrolysis of Bz-Arg-Et. The activity of human enteropeptidase was determined spectrophotometrically with Bz-Arg-Et as a substrate [8]. 20 µl α_2-macroglobulin-free enteropeptidase concentrate containing 2.5 I.U./l [7] was added to 0.9 ml 1 mM Bz-Arg-Et in 20 mM Tris · HCl buffer (pH 7.5) and the change of absorbance at 253 nm recorded at 25°C. In parallel experiments 2.5 mmol glycodeoxycholic acid was added per l substrate solution.

Results

The rapid, single-stage assay of enteropeptidase reported by us earlier [7] was found unsuitable for this work because our trypsin substrate (Z-Gly-Gly-Arg-NapNH) forms insoluble precipitates with bile acids. We therefore

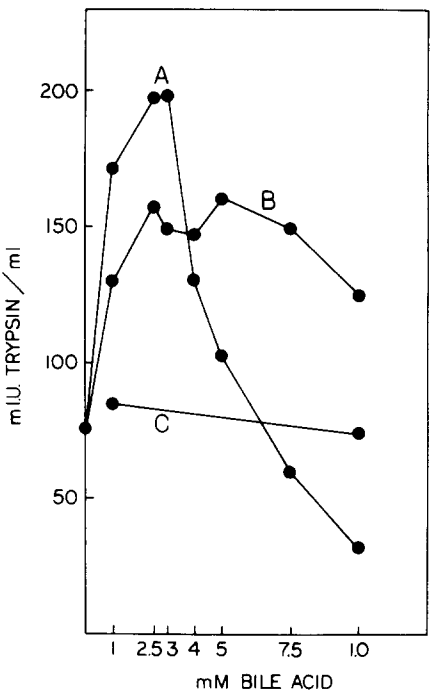

Fig. 1. Effect of glycodeoxycholic (A), glycocholic acid (B) and NaCl (C) on enteropeptidase-catalyzed trypsinogen activation. The assay mixtures contained 1.3 μI.U. [7] of enteropeptidase, 10 μmol trypsinogen/l and increasing concentrations of bile acids or NaCl as indicated on the abscissa. Activity is recorded as mI.U. trypsin generated/ml, as described under Materials and Methods.

employed a modification of the customary Kunitz two-stage procedure, retaining our highly sensitive, fluorogenic substrate for the second stage of our assay.

Effect of individual bile acids. There are two groups of major bile acids in human bile which differ from one another in their optimum concentration at which they interact with the enteropeptidase-trypsinogen system (Fig. 1). The first (A) includes glycodeoxycholic, taurodeoxycholic, glycochenodeoxycholic and taurochenodeoxycholic acid. Optimum concentration of these bile acids in our system was approximately 2.5 mM, as reported for glycodeoxycholic acid by Hadorn et al. [2] and illustrated in Fig. 1 (A). Higher concentrations diminished enhancement of enteropeptidase activity and eventually caused inhibition. The second group (B), glycocholic and taurocholic acid, acted optimally at concentrations between 2.5 and 10 mM. A mixture of all major bile acids in physiological proportions [9] exhibited a concentration optimum of about 3.5 mM under routine assay conditions. Higher concentrations decreased enhancement of enteropeptidase activity as shown for glycodeoxycholic acid.

Effect of bile acids on enteropeptidase-catalyzed hydrolysis of Bz-Arg-Et. Human enteropeptidase, like the porcine enzyme [11], catalyzes the hydrolysis of Bz-Arg-Et. Addition of bile acids (glycodeoxycholic acid) to this system had no effect on the reaction velocity.

Effect of ionic strength on bile acid-enhanced activity of enteropeptidase. Table I illustrates the increase in enteropeptidase activity elicited by glycodeoxycholic acid and the effect of increasing ionic strength on this interaction.

TABLE I

EFFECT OF IONIC STRENGTH ON BILE ACID ENHANCEMENT OF ENTEROPEPTIDASE ACTIVITY

All incubation mixtures contained 2.5 mmol NaCl or 2.5 mmol glycodeoxycholic acid/10 μmol trypsinogen/1/5 · 10^{-4} mI.U. [7] enteropeptidase. Ionic strength was increased by adding water, 10 or 50 mM Tris · HCl and increasing amounts of NaCl in 50 mM Tris · HCl to give a total volume of 1.05 ml in the first stage reaction mixture described under Materials and Methods.

$I \times 10^2$	mI.U. Trypsin generated/ml incubation mixture		Ratio of activity in the presence/activity in the absence of glycodeoxycholic acid
	Without glycodeoxycholic acid	With glycodeoxycholic acid	
0.25	39.0	382	9.8
0.51	52.7	375	7.1
1.55	99.0	200	2.0
1.93	87.3	172	2.0
2.55	66.1	127	1.9
3.80	42.0	84	2.0
6.30	29.4	57	1.9
7.30	26.6	49	1.8

It can be seen that the highest reading as well as the greatest increase in activity due to glycodeoxycholic acid was obtained at the two lowest ionic strengths. In all subsequent experiments with $I > 0.005$, the absolute readings for enteropeptidase activity decreased progressively, but the ratio of activity in the presence of glycodeoxycholic acid/activity in the absence of glycodeoxycholic acid now remained constant at a value of about 2.

Effect of anions on enteropeptidase activity. To test the influence of different anions on the activity of enteropeptidase, the enzyme was assayed as described under Materials and Methods in the presence of increasing concentrations of $NaClO_4$, NaCl and Na_2SO_4. Fig. 2 presents a comparison of the inhibitory effect of increasing isoionic concentrations of these salts. Perchlorate was the least, sulfate the most inhibitory anion tested.

Effect of monovalent cations. In a series of analogous experiments the influence of monovalent cations, Li^+, Na^+, K^+ and Cs^+ was investigated; all salts used were chlorides. Fig. 3 shows that the effect of these cations on enzyme activity did not vary greatly from one metal to another and was not nearly as striking as that of the anions tested.

Kinetic experiments. Michaelis-Menten constants for enteropeptidase-catalyzed activation of trypsinogen were determined by means of Lineweaver-Burk plots. Trypsinogen concentrations used were 0.1—10 μM and the amount of enzyme 2.5—7 · 10^{-4} mI.U. [7]. The slope of the plots was calculated by linear regression analysis of the experimental data.

The ionic strength-dependence of K_m is illustrated in Fig. 4 which shows two plateaux at K_m 1.2 · 10^{-5} and 2.5 · 10^{-5} M between I: 0.019—0.0255 and 0.038—0.063, respectively.

The influence of ionic strength on the bile acid effect in enteropeptidase-catalyzed trypsinogen activation was studied by means of Lineweaver-Burk plots derived from experiments run at different ionic strengths and in the

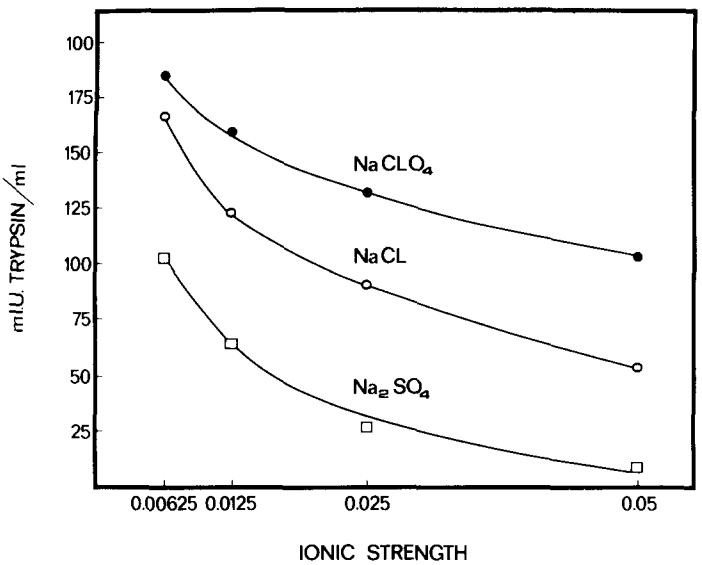

Fig. 2. Effect of increasing isoionic concentrations of different anions (ClO_4^-, Cl^-, SO_4^{2-}) on activity of human enteropeptidase. Enteropeptidase assays were performed in the presence of 4 different concentrations of $NaClO_4$, NaCl and Na_2SO_4, respectively. Appropriate additions of these salts were made to the assay mixtures to give isoionic increases as listed in the abscissa. Enzyme concentration for all experiments: 1.3 μI.U./ml [7].

absence or presence of 2.5 mM glycodeoxycholic acid, as outlined in the preceding section. Data obtained at very low ionic strength (I approximately 0.0026) are shown in Fig. 5. It can be seen that V is higher in the presence of

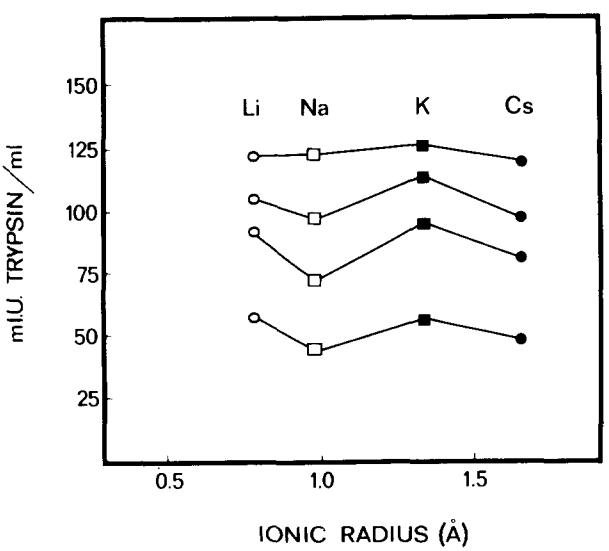

Fig. 3. Effect of increasing isoionic concentrations of different alkali metals (Li^+, Na^+, K^+, Cs^+) on activity of human enteropeptidase. Isoionic concentrations of different metals are connected by horizontal lines. Ionic strength values as in Fig. 2. Line at the top of Fig. 3 represents the lowest, line at the bottom of Fig. 3 the highest ionic strength. Enzyme concentration in all experiments: 0.9 μI.U./ml [7].

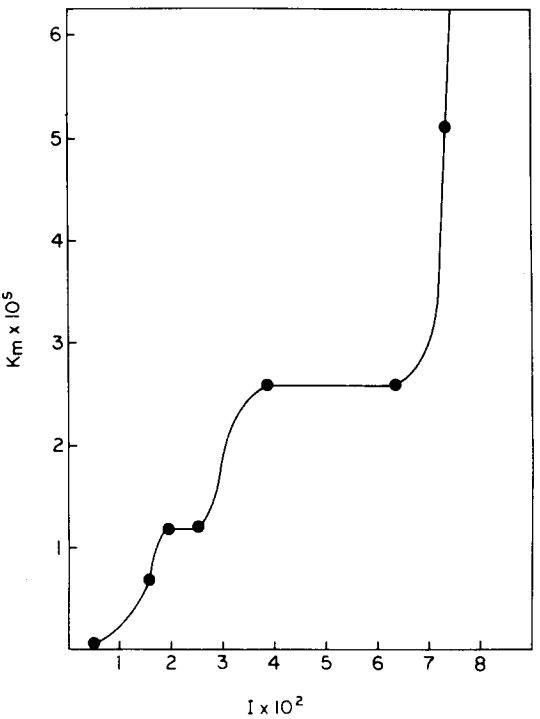

Fig. 4. Effect of ionic strength on K_m of enteropeptidase-catalyzed activation of trypsinogen. Each point on the illustration was obtained by assay of a constant amount of enteropeptidase with 5 different concentrations of trypsinogen varying from 0.1 to 10 μM. The ionic strength of the assay mixture was increased for each point on the curve by appropriate addition of NaCl. Michaelis-Menten constants were calculated by regression analysis of the Lineweaver-Burk plots of the assay results.

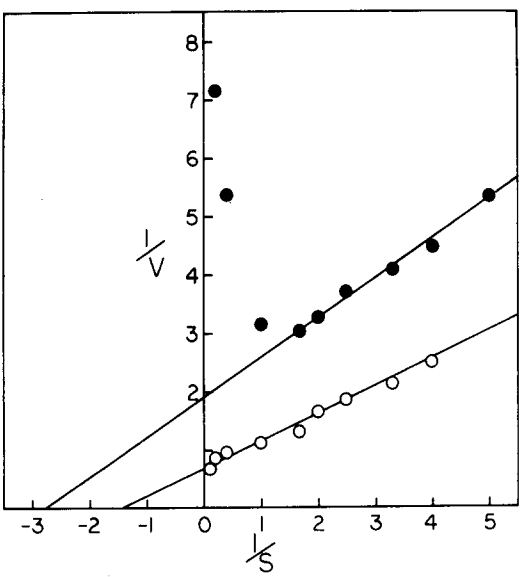

Fig. 5. Effect of glycodeoxycholic acid on K_m of enteropeptidase activation of trypsinogen at very low ionic strength ($I \approx 0.0026$). Buffer of standard assay replaced by water, pH 5.3. Filled circles, no glycodeoxycholic acid, (K_m, $3.4 \cdot 10^{-7}$ M); empty circles, 2.5 mM glycodeoxycholic acid (K_m, $6.2 \cdot 10^{-7}$ M). S, μM.

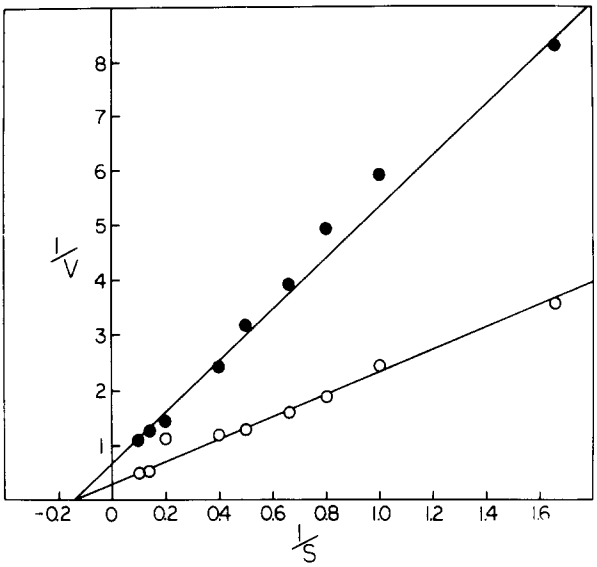

Fig. 6. Lineweaver-Burk plots illustrating effect of bile acids on enteropeptidase-catalyzed trypsinogen activation at ionic strengths between I: 0.0155 and I: 0.073. Filled circles, no glycodeoxycholic acid (K_m, $0.7 \cdot 10^{-5}$ M); empty circles, 2.5 mM glycodeoxycholic acid (K_m, $0.65 \cdot 10^{-5}$ M). Experiment in illustration carried out under standard conditions varying trypsinogen concentrations between 0.75 and 10 μM. I, 0.0155; S, μM.

glycodeoxycholic acid than in its absence and K_m is increased in the presence of bile acid. Fig. 6 demonstrates the effect of ionic strength above 0.0155 on the kinetics of this reaction. It will be noted that V again is greater in the presence than in the absence of glycodeoxycholic acid. K_m on the other hand, now remains the same in the absence or presence of bile acid, in contrast to the conditions illustrated in Fig. 5. It can be seen also in Fig. 6 that an increase in ionic strength results in an increase of K_m, as would be expected from data presented in Fig. 4.

Discussion

The results presented here confirm earlier reports that bile acids increase the velocity of enteropeptidase-catalyzed activation of trypsinogen [2,10] and contradict findings reported recently by Barns et al. [4].

Previous investigators did not report differences between individual bile acids with respect to their ability to enhance enteropeptidase activity. We have found that there are two groups of bile acids, derived from cholic acid on one hand, and deoxy- or chenodeoxycholic acid on the other, which differ in their "efficiency profiles" and optimum concentrations, as illustrated in Fig. 1. Deoxy- and chenodeoxy bile acids were more effective in enhancing enteropeptidase activity than cholic acid derivatives. It is of interest in this connection to point out that deoxy bile salts were found to be superior to cholate and glycocholate in stimulating lactosyl-ceramide β-galactosidase activity [11]. Furthermore, a recent study of the effect of bile salts on enzymatic activity of bovine lipase by Rathelot et al. [12] furnished a profile almost indistinguishable from

that shown in our Fig. 1. The behavior of these enzyme systems in the presence of bile salts suggested the possibility that bile salts may enhance enteropeptidase activity by their detergent action. However, activity was diminished rather than enhanced by addition of Triton X-100 (0.01—2%) to the reaction mixture. These results are at variance with a recent report [4] in which a 10% increase of enteropeptidase was observed by similar concentrations of the detergent. Other detergents such as sodium dodecyl sulfate caused precipitation of the trypsin substrate in the second stage of the assay and were not suitable for these experiments. Trypsin activity itself was not affected by the presence of bile acids (bile acid concentration in the incubation mixture, 25 μM).

The ionic strength dependence of rat and pig enteropeptidase has been pointed out in several recent reports [5,6], but no such study has been described for the human enzyme. Fig. 4, which illustrates this relationship for human enteropeptidase, shows a plateau of constant K_m between I: 0.038—0.063. A second plateau may exist between I: 0.019 and 0.026, but the narrow range and relatively poor reproducibility of such small differences permit other interpretations of these data. However, two such plateaux have been reported for the porcine enzyme. They are thought to reflect the presence of two subsites on the enzyme with different sensitivities towards the ionic environment [6].

Our results show that the effect of ionic strength on enzyme activity depends not only on the magnitude of this parameter, but also on the nature of the anion (but not of the monovalent cation) (Figs. 2 and 3). It is conceivable that such anions may interact specifically with the cationic specificity site of the enzyme which recognizes the tetraaspartyl sequence of trypsinogen, and increase dissociation (k_2) of the Michaelis complex (ES). "Blocking" of the specificity site of the enzyme by anions could be considered the counterpart to the interaction of lanthanide cations with the anionic tetraaspartyl sequence of the substrate which also results in decreased enzyme activity [13].

The influence of ionic strength on the augmentation of enteropeptidase activity by bile acids is illustrated in Table I. An 8—10-fold increase in enzyme activity was obtained at very low ionic strength, whereas at ionic strength higher than 0.0051 the increase remained approximately 2-fold over the entire range of ion-concentrations tested (0.0155—0.073). This apparent anomaly is related to the trypsinogen concentration used in these experiments and is explained below.

At ionic strengths between 0.0155 and 0.073 K_m increased as shown in Fig. 4. Addition of bile acid, however, did not significantly change K_m, but increased V about 2-fold in each of the paired experiments. This relationship, in accordance with Michaelis-Menten kinetics for single substrates, suggests that bile acids decrease dissociation (k_2) of the Michaelis complex (ES), perhaps by facilitating interaction of the enzyme specificity subsite with the polyaspartyl group of trypsinogen. The absence of a bile acids effect on enteropeptidase-catalyzed hydrolysis of Bz-Arg-Et is consistent with this view.

The Lineweaver-Burk plots shown in Fig. 5 illustrate the kinetics of enteropeptidase-catalyzed trypsinogen activation at very low ionic strength (approx. 0.0026) in the absence and presence of bile acid. The data indicate "substrate inhibition" of the enzyme between 2.5 and $10 \cdot 10^{-6}$ M trypsinogen in the

absence (A), but not in the presence (B) of the bile acid. Since all experiments summarized in Table I were carried out at $10 \cdot 10^{-6}$ M trypsinogen (highest inhibition by substrate in the absence of bile acid), these findings offer an explanation for the exaggerated effect of bile acids on enteropeptidase at very low ionic strength.

The anomalous kinetics at "high" concentrations of trypsinogen and very low ionic strength (Fig. 5) might be explained by reaction of two molecules of substrate with one molecule of enzyme, whereby one molecule of trypsinogen would react normally with the catalytic site, the second through its polyanionic $(Asp)_4$ group with the positively-charged specificity site of the same enzyme molecule, thus reducing the formation of normal ES complex. The presence of a critical concentration of other anions (Cl^-, ClO^-_4, SO_4^{2-}) or bile acids may prevent this, as indicated by our findings. There are other explanations for our observations which should be considered. Thus, we attempted to determine whether the effect of increased ionic strength on enzyme activity may reflect increasing aggregation of trypsinogen [14]. However, sucrose density gradient sedimentation experiments in a Beckman L-5 ultracentrifuge with trypsinogen solutions corresponding to those employed in our kinetic studies did not furnish any evidence in support of such a hypothesis. The possibility that ionic strength or bile acids or both may affect not only the conformation of the substrate, but also that of the enzyme itself, should likewise be kept in mind. Such studies, unfortunately, are severely handicapped by the paucity of human enteropeptidase.

Acknowledgement

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