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RAT ENTEROKINASE: THE EFFECT OF IONS AND THE LOCALIZATION IN THE INTESTINE

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SUMMARY

1. The effects of divalent metal ions, ionic strength and pH on the activity of rat enterokinase (enteropeptidase, EC 3.4.4.8) were studied. No important activators were found, and most of the metal ions studied inhibited the activity. The ionic strength markedly influenced the enterokinase activity.

2. Using incubation conditions which as far as possible fulfilled the criteria for optimal enterokinase activity, we studied the distribution of the enzymic activity along the small intestine, in the different layers of the intestinal wall and on the subcellular level.

3. Enterokinase activity was present along most of the length of the small intestine, highest activities being found in the duodenum. It was present in the villi but essentially absent from the crypts. No activity was found in the Brunner glands. The subcellular localization was in the brush borders of the villous epithelial cells.

4. That enterokinase is a brush-border enzyme means the discovery of a new functional property of this organelle, since this enzyme differs fundamentally from other brush-border enzymes both as to the type of substrate and the point of action in the sequence of digestive events.

INTRODUCTION

Enterokinase (enteropeptidase, EC 3.4.4.8) initiates the pancreatic digestion of protein by proteolytic conversion of trypsinogen into trypsin, which in turn activates the other proteolytic proenzymes from the pancreas, chymotrypsinogens and procarboxypeptidases. Thus enterokinase is a key enzyme for the utilization of dietary protein, the absence of which implies severe clinical symptoms[1,2].

That freshly secreted pancreatic juice contains no active proteases was shown a century ago by KÜHNE[3] and HEIDENHAIN[4]. Later SCHEPOWALNIKOV[5], in PAVLOV's laboratory, discovered that such juice was activated by an intestinal factor which he named enterokinase, and PAVLOV[6] called it "a ferment of ferments" to stress its superior catalytic function. However, it was not until 1939 that KUNITZ[7], in an

important work on the mechanism of formation of trypsin from trypsinogen by means of enterokinase, clearly established that this process is truly enzymic in the modern sense of this word. KUNITZ worked with a partially purified enterokinase from swine duodenal contents. Later YAMASHINA[8] further purified enterokinase and showed that this enzyme has a high carbohydrate content. He also showed that the trypsinogen activation by enterokinase in chemical terms means that a valylpeptide is split off from the trypsinogen molecule[9]. A similar, if not identical, reaction occurs on autocatalytic activation of trypsinogen[10].

The general concept is that enterokinase is formed in the small intestinal wall and secreted therefrom with the intestinal juice into the lumen of the gut. Consequently, most interest in enterokinase has been directed towards the conditions in the juice, and the enzyme has been studied in the duodenal juice as an indicator of the state of the intestinal mucosa in gastrointestinal diseases and after pharmaceutical and radiation therapy, especially by Russian scientists. To our knowledge, no study has been carried out on the site of enterokinase formation in the intestinal wall, although precise information on the localization of the enzyme should improve our understanding of the physiological activation of trypsinogen in the gut. We have therefore studied the localization of enterokinase in the rat at different levels: the quantitative localization along the intestine, the quantitative localization within different layers of the intestinal wall, and the subcellular localization. Preliminary reports on the localization have been published[11–13].

During experiments on the subcellular localization of enterokinase we observed that EDTA, necessary for the isolation of the brush borders, inhibited the activation of trypsinogen by enterokinase. Recently it was reported that low concentrations of calcium ions activate enterokinase[14,15], which suggested that EDTA might inhibit by complex formation with calcium ions. After some preliminary experiments, however, it became clear that a more systematic investigation of the effect of ions had to be undertaken to find the optimal ionic conditions for the assay of enterokinase activity to be used in the localization experiments. The results of this study will be reported first in this paper.

MATERIALS AND METHODS

Animals

Rats (200–350 g) of Wistar strain, either sex, were used. They were starved overnight if not otherwise stated. They were killed by excision of the heart during light ether anesthesia. Only the duodenum was used, if not otherwise stated, for two reasons: firstly, the duodenum is the part of the intestine to which the trypsinogen of the pancreatic juice is presented; secondly, the highest activities of enterokinase were found in the duodenum.

Tissue preparations

Effect of ions. Mucosal homogenates and purified brush border preparations from the duodenal mucosa were used as the source of enterokinase for studies on the influence of different amounts of enzyme and substrate and on the effect of ions on the enterokinase activity. The mucosal homogenates were made by homogenizing mucosal scrapings with 10 volumes of water (Ultra-Turrax homogenizer), and they

were then usually dialyzed against 0.005 M sodium maleate buffer (pH 6.0), or—when the effect of metal ions was to be tested—first against 0.005 M sodium EDTA (pH 7.0) and thereafter against maleate buffer. The purified brush border preparations were made as described below.

Localization along the intestine. To determine the distribution of enterokinase activity the whole small intestine (including the duodenum) was cut out from rats that had been fasted for 16 h. The intestine was cut into pieces each 6 cm long, rinsed in saline, homogenized *in toto* in water, and analyzed for enterokinase activity and protein.

Localization in the villi and crypts. Tissue from different parts of the villi, crypts and submucosa was obtained by a microdissection technique previously described in detail in connexion with studies on the localization of other intestinal enzymes[16–18].

This technique involves horizontal cutting of frozen pieces of intestinal wall in a cryostat in such a way that serial sections of only cross-sectioned villi or crypts are obtained. The sections were collected in groups and homogenized except for the last section in each group which was used for microscopical identification. The homogenates were used for enzyme analyses and for determination of protein to allow the calculation of the specific enzyme activities in each homogenate.

In the present experiments, duodenum was used from rats that had been fasted for different periods (3–24 h). Some of the animals were killed by a blow on the head instead of the routine excision of the heart during ether anesthesia. After perfusion of the duodenum with saline (1.3 ml/cm of intestine) it was opened and residual mucus was removed by careful blotting with a piece of dry soft paper. Pieces of intestinal wall from different parts of the duodenum were sectioned and the homogenates were made in water.

In a specially designed experiment the whole length of the duodenum from the pylorus and distally into the jejunum was cut into pieces after rinsing and microdissected. The purpose was to exclude (or find) that at some special small area of the duodenum the localization of enterokinase activity in the wall differed fundamentally from the distribution usually obtained. Therefore, the trimmings of the edges of the frozen pieces were limited to a minimum (about 1 mm for each side).

Subcellular localization. Brush borders were isolated essentially as described by MILLER AND CRANE[19]. Some minor modifications have been taken from the methods published by PORTEOUS[20] and FORSTNER *et al.*[21].

The duodenum was cut out from 5–6 rats and rinsed thoroughly with ice-cold 0.9% NaCl (25–40 ml) from a burette. Excess luminal fluid together with residual mucus was removed by gently stroking the opened intestine lengthwise with the edge of a microscope slide, and the mucosa was then scraped off with another slide. Subsequent steps in the procedure were carried out at 0–5°. The mucosa (1.9–2.1 g wet weight) was suspended in 120 ml of 0.005 M sodium EDTA (pH 7.0) and homogenized with a top-drive macerator (Sorvall Omnimixer) operating at 3500 rev/min for 35 sec. Carefully controlled conditions of homogenization were essential for the result. The homogenate (90 ml) was filtered through No. 25 bolting silk (100 μ square-mesh nylon cloth in later experiments). After centrifugation at 600 $\times$ g for 10 min, the sediment was washed 3–4 times by suspension and resedimentation in a smaller volume (30 ml) of 0.005 M EDTA (pH 7.0). Finally, the brush borders were

suspended in a suitable volume of 2.5 mM EDTA (pH 7.0). This preparation (dialyzed as specified below) will be referred to as crude brush borders.

When further purification of brush borders was required the final sediment of crude brush borders was treated with NaCl, which gives a flocculation of nuclei, disintegrated nuclear material and other cellular debris but not of brush borders.

The crude brush border sediment was suspended in 30 ml of 0.120 M NaCl in 0.005 M EDTA (pH 7.0), and allowed to stand for 30 min. The aggregated material was then removed by low-speed centrifugation. The supernatant, containing the brush borders, was passed once through 25 μ nylon cloth to remove any larger contaminants remaining in the supernatant. The filtrate was centrifuged at 600 $\times$ g for 10 min and the brush border sediment was finally suspended in a suitable volume of 2.5 mM EDTA (pH 7.0). This preparation (dialyzed as specified below) will be referred to as purified brush borders. The degree of purity was briefly checked with a phase contrast microscope at each step of preparation.

Sediments during preparation of brush borders from duodenum seem to be looser and more illdefined than sediment from jejunum and ileum, and therefore special care had to be taken not to discard part of the sediment with the supernatants. This difference in properties may in part explain why we had some initial difficulties in making good brush border preparations from duodenum although in a few experiments we obtained good preparations from the rest of the intestine.

In preliminary experiments it was found that EDTA interfered seriously with the assay of enterokinase activity. EDTA, therefore, had to be removed in all steps (original mucosal homogenate, different supernatants and crude and purified brush border preparations) by dialysis before analysis could be performed. The dialyzing procedure was the same both in the subcellular localization experiments and when the purpose was to prepare purified brush borders as an enzyme source for studies of the effect of ions on the activation of trypsinogen by enterokinase. Because brush border preparations were wanted essentially free from divalent metal ions, when ion studies were intended, samples were first dialyzed against 0.005 M sodium EDTA (pH 7.0) for at least 3 h, and EDTA was thereafter removed by dialyzing against 10 l 0.005 M sodium maleate buffer (pH 6.0) at 4° for at least 16 h with one exchange of the outer medium after a few hours. A sample of the final outer medium was saved for use in enzyme dilutions.

Dialysis was also needed because we observed that EDTA depressed the color reaction in the method for protein determination according to LOWRY *et al.*[22,23].

Chemicals

Bovine trypsinogen from pancreas (1 $\times$ crystallized) was purchased from BDH, Poole, England. According to the seller, the preparation was absolutely free of trypsin inhibitor but contained up to approximately 50% $MgSO_4$. In preliminary experiments trypsinogens from other companies were also tested (Fluka, Sigma, Seravac), but in none was a higher potential trypsin activity found.

$MgSO_4$-free trypsinogen was obtained by dialyzing the commercial trypsinogen, 4 mg/ml 0.001 M HCl, against 0.001 M HCl which prevents autoactivation during the desalting procedure. The dialyzed trypsinogen could be stored for at least two days at 0° without any significant autoactivation.

Bovine crystallized trypsin of high purity was obtained from Novo Industri, Copenhagen, Denmark.

Metal salts were of analytical grade, as were other reagents used in preparing the different solutions.

Methods

Assay of enterokinase activity. Enterokinase has a very high specificity for trypsinogen[9] and this physiological substrate is in fact the only one known for the enzyme. This means that the determination of enterokinase activity has to be performed in two steps as originally described by KUNITZ[7]. Trypsinogen is first incubated with the enterokinase-containing solution and then samples are removed from the activation mixture at suitable time intervals and analyzed for the amount of trypsin activity produced. In his method, KUNITZ took into account the complications due to the action of trypsin formed, *i.e.* chiefly autocatalytic activation and formation of inert protein from trypsinogen. Both processes were much reduced by using an extremely low trypsinogen concentration, and the formation of inert protein was further suppressed by activation at pH below 6.0.

The standard assay procedure for determination of enterokinase activity described below is a modification of a method used by HADORN *et al.*[1] which in turn is based on the method of KUNITZ.

Activation mixture. 0.1 ml of 0.1 M sodium maleate buffer (pH 6.0), 0.1 ml enterokinase-containing solution, and 1.2 ml distilled water were mixed and prewarmed at 25°, the incubation temperature. Incubation was started by the addition of 0.1 ml trypsinogen solution (2 mg trypsinogen per ml 0.005 M HCl). The commercial trypsinogen was not dialyzed before use. After a suitable time of activation (not more than 60 min) the determination of the amount of trypsin formed was immediately started by the addition of an aliquot of the activation mixture to a tube with the reagents for the assay of trypsin activity. Different dilutions of each sample were usually analyzed to check the proportionality and to obtain an optimal reading value. A blank tube was run with omission of trypsinogen. This tube would also reveal any trypsin accidentally present in the enzyme sample. In separate tubes it was also always checked that the trypsinogen itself was free from trypsin and that no autoactivation of the trypsinogen occurred during the experimental procedure. Analysis of samples from an enterokinase stock solution (a pooled homogenate stored in the freeze-box) was further used to check the reliability of the assay system.

Trypsin determination. The substrate used was benzoyl-DL-arginine *p*-nitroanilide·HCl which on hydrolysis gives the yellow *p*-nitroaniline. Benzoyl-DL-arginine *p*-nitroanilide·HCl was dissolved in a small volume of dimethylsulfoxide and then diluted with 0.05 M Tris buffer (pH 8.2) containing 0.02 M $CaCl_2$, to give a substrate concentration of 0.001 M[24]. Incubation at 25° was started by the addition of 0.1 ml activation mixture to 0.5 ml substrate buffer solution, and stopped after 60 min by the addition of 0.1 ml 30% (v/v) acetic acid. After centrifugation to remove turbidity, the color was read with a Vitatron filterphotometer at 409 nm. A standard curve with different amounts of *p*-nitroaniline was made for the calculation of the enterokinase activity. A unit of trypsin is the activity that hydrolyzes 1 μmole of benzoyl-DL-arginine *p*-nitroanilide·HCl per min. 1 unit of enterokinase is the activity that activates 1 unit of trypsin per min.

Assay of alkaline phosphatase activity. Alkaline phosphatase activity was measured with a modification of the method of BESSEY, LOWRY AND BROCK[25] using *p*-nitrophenyl phosphate as substrate, in the presence of magnesium, zinc and cobalt ions as activators[26]. Readings were performed in a microcuvette with a Vitatron filterphotometer. This instrument was also used in the assays presented below. One unit of alkaline phosphatase is defined as the activity that hydrolyzes 1 μmole substrate/min at 37°.

Assay of disaccharidase activity. Disaccharidase activity was measured with the method of DAHLQVIST[27]. Turbid reaction mixtures were centrifuged immediately before reading. 1 unit of disaccharidase is defined as the activity that hydrolyzes 1 μmole substrate/min at 37°.

Determination of protein. Protein was measured with the method of LOWRY *et al.*[22] as modified by EGGSTEIN AND KREUTZ[23]. Human serum albumin was used for preparation of the standard curve. When limited amounts of material were available, as in the micro-dissection experiments, the reaction volumes were reduced 10 times. Good proportionality and duplicates could still be obtained with this micromodification (*cf.* MOOG AND GREY[28]).

RESULTS

Influence of enterokinase and trypsinogen concentrations

According to KUNITZ[7], the formation of trypsin from trypsinogen by enterokinase follows the course of a first-order reaction. However, in the initial phase of the activation process (corresponding to the conversion of a small part of the total amount of trypsinogen present in the activation mixture) the kinetics are practically of zero order. A direct proportionality was found between the amount of enterokinase present and the amount of trypsin produced (Fig. 1), and time curves showed a linear relation between time of activation and the increase of trypsin activity. With crude mucosal homogenates as source of enterokinase the proportionality curve did not always pass exactly through the origin.

The curve in Fig. 1 was obtained with the standard concentration of trypsinogen (0.13 mg/ml activation mixture). There is a linear relation up to an absorbance

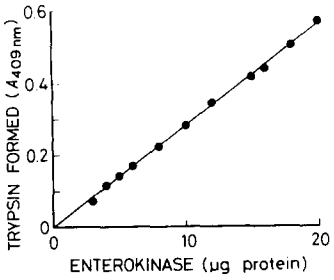

Fig. 1. Proportionality curve for enterokinase activity. Amount of trypsin formed *versus* enterokinase concentration (expressed as the total amount of protein added to the activation mixture). A brush border preparation was used as the source of enterokinase. Standard assay procedure. Activation time 60 min.

of at least around 0.600. Proportionally too little trypsin was formed if greater amounts of enterokinase were added to the activation mixture. The linear range was considerably shortened if the concentration of trypsinogen was reduced to half and the rate of activation was less rapid.

Trypsin formed by the action of enterokinase will always tend to produce more trypsin by autocatalysis. However, with the standard conditions used for assay, no important autocatalysis occurred, as concluded from time curve studies and measurements of the "enterokinase" effect exerted by pure bovine trypsin. Concerning the potency of enterokinase and trypsin, respectively, to activate trypsinogen, a certain amount of enterokinase (mg brush border protein) was much superior to the same amount of crystallized bovine trypsin (mg protein) as an activator under the standard conditions used.

Influence of metal ions

Table I shows the effect of various bivalent metal ions on the activation of trypsinogen by enterokinase. All metals were added as their chlorides except Mn^{2+},

TABLE I

EFFECT OF BIVALENT METAL IONS ON ENTEROKINASE ACTIVITY

Metal ion added	*Concentration in the activation mixture (10^{-3} M)*	*Enterokinase activity (%)*
0	0	100
Co^{2+}	1.0	102
Zn^{2+}	1.0	94
Mg^{2+}	1.0	51
Ca^{2+}	1.0	39
Ba^{2+}	1.0	53
Mn^{2+}	1.0	45

which was added as the sulfate, and the enzyme sample was pre-incubated for 20 min with the metal ion before trypsinogen was added. Concentrations of metal salts were made 0.001 M in the activation mixture. A brush border preparation was used as source of enterokinase. Co^{2+} had no effect at all, and Zn^{2+} had a very small negative effect. All other metal ions tested, including Ca^{2+}, inhibited the enterokinase activity to a considerable extent. The inhibition really took place in the activation (enterokinase) step and not in the trypsin determination step, because in separate experiments it was checked that the assay of trypsin was not influenced by the amount of metal ions transferred from the activation mixture with the sample used for trypsin determination. The lack of inhibitory effect on trypsin could also be predicted at least for calcium ions because these are known to activate trypsin (the trypsin assay system has a concentration of around 0.017 M with respect to calcium ions).

Co^{2+} and Zn^{2+} had a slight activating effect in lower concentrations (20–30% increase in enterokinase activity at an optimal concentration of about 0.1 mM in the activation mixture), but Ca^{2+} did not activate significantly at any concentration tested (Fig. 2). Co^{2+} and Zn^{2+} together did not increase the small activating effect more than each of these ions did separately. A stronger activation of enterokinase activity by Co^{2+} and Zn^{2+} was observed when the pH of the activation mixture was

increased (pH 6.8, 7.4, 7.8, Tris–maleate buffer being used instead of the standard maleate buffer). That this was not due to promotion of autocatalytic transformation of trypsinogen was checked in control experiments, in which crystallized bovine trypsin instead of enterokinase was added at zero time to activation mixtures with different pH.

The results presented above are from experiments in which brush border preparations were used as source of enterokinase. With mucosal homogenates the results were slightly modified although essentially the same. Co^{2+} or Zn^{2+} depressed the enterokinase activity at 0.001 M concentration and did not activate significantly at any concentration. The inhibitory effect of the other ions was more pronounced.

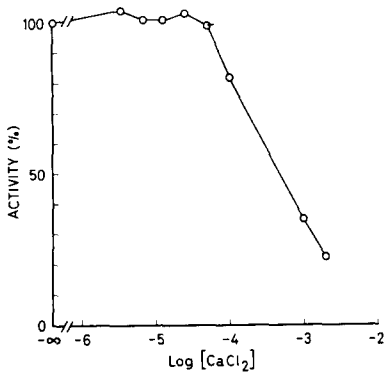

Fig. 2. Effect of $CaCl_2$ on the enterokinase activity. The concentration of $CaCl_2$ (M) refers to the concentration in the activation mixture. A brush border preparation was used as source of enterokinase. The activity in the control (no $CaCl_2$ added) was arbitrarily set at 100.

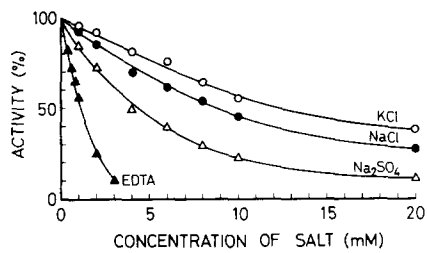

Fig. 3. Influence of different salts and sodium-EDTA on the enterokinase activity. The activity in the control (no salt added) was arbitrarily set at 100. The concentration of salt (mM) refers to the concentration present in the activation mixture.

$MgSO_4$ present in the commercial trypsinogen preparations used in the standard assay procedure may modify the effect of other ions added. Therefore, we also tested the effect of divalent metal ions on the enterokinase activity with a $MgSO_4$-free trypsinogen. The most striking effect with this dialyzed trypsinogen was that a higher activation rate was obtained, which was due to the removal of inhibitory ions. The effect of the various ions was essentially the same as with the commercial trypsinogen, and again we found no significant activating effect of Ca^{2+}.

Influence of ionic strength

The influence of different concentrations of NaCl, KCl and Na_2SO_4 on the enterokinase activity was tested both with mucosal homogenates (Fig. 3) and with brush border preparations as source of enterokinase. Similar effects were observed with both kinds of enzyme preparation. All three salts depressed the enterokinase activity, the degree of inhibition being proportional to the concentration. The effect, therefore, was not ion-specific but seemed to be an effect of the ionic strength. In preliminary experiments it had been ruled out that this effect could be exerted in

the trypsin determination step and not in the activation (enterokinase) step. As can be seen in Fig. 3, EDTA was also tested, using as enzyme source a brush border preparation that had been dialyzed against EDTA (to remove bivalent metal ions) and thereafter against diluted maleate buffer. The effect of EDTA was strongly inhibitory and seemed mainly to be due to the increasing density of charge in the activation mixture. That the inhibition could be due to complex formation with activatory bivalent ions is not to be expected because metal ions present in the enzyme preparation had been removed before analysis. Moreover, we found no important metal dependency for the enterokinase activity. The order of increasing inhibition exerted by the different compounds agrees well with the values of ionic strength that can be calculated for each of them.

Since the ionic strength of the activation mixture had such a great influence on the enterokinase activity, it could be expected that the determination of the pH optimum for the activation of trypsinogen by enterokinase would be influenced by the buffer used. In preliminary experiments, it was also found that doubling the standard concentration of buffer (6.7 mM in the activation mixture) considerably depressed the activation rate. The pH optimum was therefore determined using two kinds of maleate buffer, one with constant molarity (6.7 mM in the activation mix-

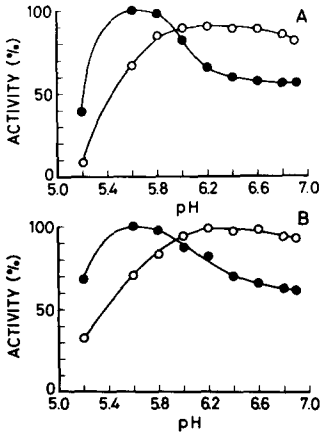

Fig. 4. pH optimum curves for the enterokinase activity with maleate buffer of isoionic (○—○) and non-isoionic strength (●—●). Source of enzyme: mucosal homogenate (A) and brush border preparation (B). The activities at different pH within each subfigure are given as percent of the highest activity obtained with either of the two kinds of buffer used.

tures), which means increasing the ionic strength with increasing pH, and another buffer with isoionic strength but then with varying molarity (approximatively 6.7 mM in activation mixtures of pH 6.0).

The use of the non-isoionic buffer gave a pH optimum at 5.6–5.8 (Fig. 4). No corresponding optimum was found with the buffer of isoionic strength, but instead there was a broad maximal plateau from around 6.0 to 6.8. Therefore, the pH optimum found with the non-isoionic buffer was rather the effect of ionic strength than of pH and this finding further illustrates the great sensitivity of the enterokinase activity to changes in ionic strength.

Localization along the intestine

Significant enterokinase activity was present along the whole length of the small intestine except for the most distal part of the ileum. Maximal specific activities were found in the duodenum, but there was no pronounced decrease in the jejunum and proximal ileum.

Localization in the villi and crypts

Morphological examination of pieces from the duodenum taken for sectioning revealed that the villi were somewhat shorter than jejunal villi although the total wall was as thick as or thicker than in the jejunum. The duodenal villi were oval in cross-section as are jejunal villi. Brunner glands were not a regular finding in the submucosal tissue of all pieces taken for micro-dissection.

The localization of enterokinase activity within the duodenal wall was studied with pieces from all parts of the duodenum and with intestine from several different animals. One or more of the following enzymic activities were usually included for comparison: alkaline phosphatase, trehalase, sucrase, maltase and isomaltase. In one experiment (illustrated in Fig. 5) all these enzymic activities were determined together with enterokinase activity. Enterokinase showed the same principal distribution as the other enzymes tested, *i.e.* practically all the activity in the villi and very little activity in the crypts. No detectable activity of enterokinase was found in the submucosal tissue of the duodenum even when the Brunner glands could be identi-

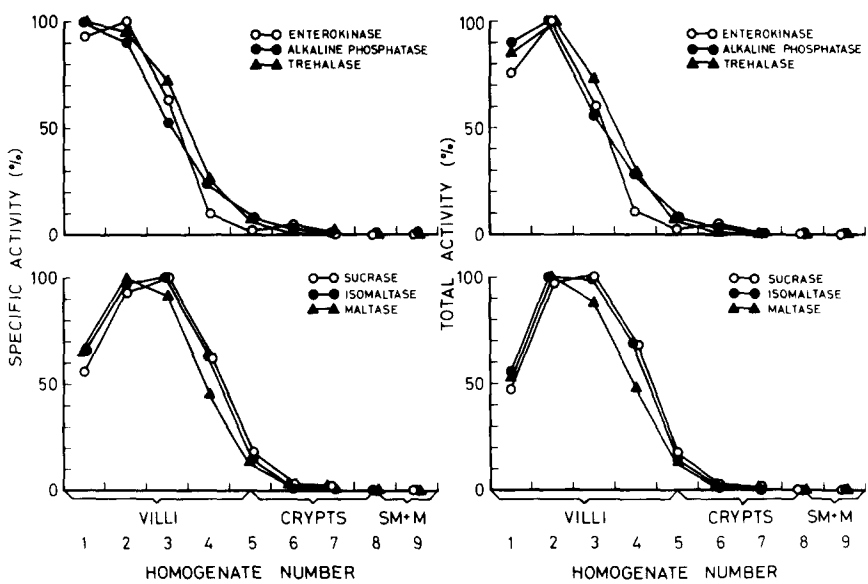

Fig. 5. Quantitative distribution of enterokinase and some other enzymic activities within the wall of the rat duodenum. The piece of intestine was taken from the middle-proximal part of the duodenum. Each homogenate contained five 20 μ thick sections, serially cut from the tips of the villi to the serosa. SM, submucosa; M, muscle tissue. The activity profiles have been put together in two groups according to similarities found in the localization pattern on the villi. The highest specific activity for each enzyme (microunits/mg protein) and highest total activity (microunits/ml homogenate) were arbitrarily set at 100, to obtain relative activities.

fied. According to characteristics in the distribution pattern on the villi, the different enzymic activities could be arranged in two groups: one group with alkaline phosphatase, trehalase and enterokinase, and another group of sucrase, isomaltase and maltase. The features of the activity profile of the latter group of enzymes were considerable activity in the basal villi, a rather broad maximum in the mid-villi, and a clear decrease in the tips of the villi. This was true both when the total and the specific activities were calculated. The quantitative distribution of enterokinase, alkaline phosphatase and trehalase activities was different, having a more apical maximum, no decrease in the tips (specific activity) and a steep gradual decline towards the crypts. Fig. 5 shows that there were approximately the same specific activities of enterokinase in the two apical fractions of the villi, but in other experiments the increase in specific activity from the crypts and up along the villi continued all the way out to the free ends of the villi.

If the ratio for the activities of one enzyme of each group (enterokinase and sucrase) is calculated for the different homogenates of the experiment illustrated in

TABLE II

ENTEROKINASE AND SUCRASE ACTIVITIES IN THE MICRODISSECTION EXPERIMENT ILLUSTRATED IN FIG. 5

Homogenate volume, 0.25 ml.

Homogenate number	*Enterokinase activity (microunits/ml)*	*Sucrase activity (microunits/ml)*	*Activity ratio enterokinase/ sucrase*
1	1.51	30.0	0.050
2	1.99	62.0	0.032
3	1.20	63.9	0.019
4	0.22	43.5	0.005
5	0.04	11.7	0.003
6	0.08	1.6	(0.050)
7	0	0.8	—
8	0	0	—
9	0	0	—

Fig. 5, a decreasing activity ratio is obtained from the tips of the villi to the crypts, reflecting the difference in the quantitative localization in the villi (Table II). A few preliminary experiments with segments from the jejunum indicate that this difference in distribution between enterokinase and sucrase activity is not so apparent in the jejunum.

The distribution profile for enterokinase activity did not change significantly when the fasting time was varied (3–24 h), but we have some, although weak, indication that there may be small variations in the shape of the activity profile between fasted and just fed animals.

In a single experiment the distribution profile for enterokinase activity (total activity) in the wall was analyzed along the whole length of the duodenum from the pylorus and distally into the jejunum (Fig. 6). The purpose of this experiment was to investigate whether the enterokinase activity in any part of the duodenum had a localization in deeper layers of the mucosa. It is apparent that the localization of

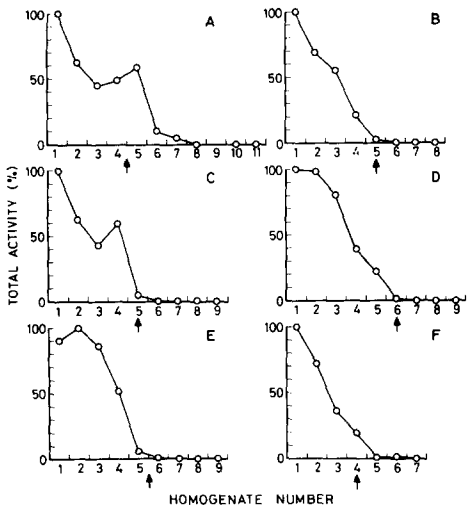

Fig. 6. Quantitative localization of enterokinase (expressed as total activity) in the villi and crypts along the whole length of the rat duodenum. The highest activity found in each experiment has been arbitrarily set at 100. A represents the most proximal part of the duodenum close to the pylorus, and F represents the most distal part of the duodenum. The arrows indicate the approximate limits between villi (left of the arrow) and crypts (right of the arrow).

enterokinase to the villi was a regular feature throughout the length of the duodenum. Sucrase and alkaline phosphatase activities were also analyzed for comparison (not illustrated), and the distribution patterns of these activities in the villi showed the same relation to the enterokinase activity in all parts of the duodenum as shown above (Fig. 5 and Table II). Curve A (Fig. 6) seems to indicate that considerable enterokinase activity was present beneath the approximate villi-crypts limit. However, alkaline phosphatase and sucrase activities showed the same peculiarity, and this phenomenon is most probably completely due to technical failure as judged from the sections taken for microscopic examination.

Enterokinase activity was thus found to have the same principal distribution profile as the disaccharidases and alkaline phosphatase. Since these enzymes on the subcellular level are located in the brush borders on the surface of the villous epithelial cells, it was logical to suspect that enterokinase could be a brush border enzyme. The

TABLE III

RESULTS FROM A BRUSH BORDER ISOLATION EXPERIMENT YIELDING CRUDE BRUSH BORDERS

The recovery is given in percent of the total activity in the original homogenate. The degree of purification is expressed as times of increase in specific activity in the brush border preparation over the original homogenate.

Enzymic activity	*Recovery (%)*	*Times purified*
Enterokinase	35	3.4
Sucrase	32	3.1
Alkaline phosphatase	34	3.3

next step in our investigation was therefore to perform a study on the subcellular localization of the enzyme.

Subcellular localization

Preliminary experiments showed that when a mucosal homogenate (Ultra-Turrax homogenizer) was centrifuged at 1500 × *g* in an ordinary laboratory centrifuge the major part (more than 70%) of the total enterokinase activity was recovered in the sediment. This finding suggested that enterokinase was not soluble but bound to particles.

Microscopical phase contrast examination of crude brush border preparations revealed that significant amounts of non-brush-border material was present together with the brush borders. The greater part of this material was apparently intact nuclei and aggregates of more or less disintegrated nuclei. The contaminants were effectively

TABLE IV

RESULTS FROM A BRUSH BODER ISOLATION EXPERIMENT YIELDING PURIFIED BRUSH BORDERS

For specification of recovery and purification see Table III.

Enzymic activity	*Recovery (%)*	*Times purified*
Enterokinase	10.9	11.0
Sucrase	13.0	13.1
Alkaline phosphatase	15.0	15.1

removed by the treatment with NaCl and subsequent low-speed centrifugation allowing the preparation of clean purified brush borders. This step, however, also cost some loss of brush borders. The individual brush borders appeared well preserved with the microvilli intact as far as could be seen at high resolution in the phase contrast microscope and in the interference microscope (Nomarski optics).

Tables III and IV give the results from two typical brush border isolation experiments, in which crude and purified brush borders, respectively, were obtained. In the first experiment the recovery was high, but the degree of purification was low; in the second experiment the reverse was true. Sucrase and alkaline phosphatase were used as brush border marker enzymes. The results show that enterokinase activity was not only found in the crude and purified brush borders but it was also present and purified to the same extent as sucrase, an exclusive brush border enzyme. We therefore conclude that the subcellular localization of enterokinase is in the brush border.

DISCUSSION

We have found no important activating effect of metal ions on the activity of rat enterokinase. This is in contrast to recent findings for enterokinase from other animal species as reported by MILSTONE *et al.*[14] and HADORN[15]. Working with partially purified hog and human enterokinase preparations, respectively, they observed a

considerable activation by Ca^{2+} (optimal concentration 0.16 and 0.5 mM, respectively). This disagreement may be due to the different animal species used; furthermore, MILSTONE *et al.* had an extremely high ionic strength in their activation mixture, which makes a comparison difficult. That Ca^{2+} at higher concentration depress the activation of trypsinogen by enterokinase is in agreement with previous findings by MCDONALD AND KUNITZ[29], MILSTONE *et al.*[14] and HADORN[15]. This inhibition, as well as that exerted by other metal ions, may partly be unspecific, *i.e.* be due to an increase in ionic strength when ions are introduced into the activation mixture. Possibly, therefore, some activating effect of Co^{2+} and Zn^{2+} is hidden by the concomitant rise in ionic strength.

Although a higher activation rate can be obtained with the same amount of enterokinase if $MgSO_4$-free trypsinogen is used instead of commercial trypsinogen, we do not usually dialyze the latter before use. The use of $MgSO_4$-free trypsinogen seems to reduce the ionic strength of the activation mixture to such an extent that the system becomes sensitive to very small differences in ionic strength between different samples. Moreover, frequent dialyzing is a tedious procedure.

The enterokinase activity was strongly depressed by a rise in the ionic strength of the activation mixture. Hence the ionic strength should be kept low by the use of a weak buffer in the activation mixture and by avoiding the addition of samples of high ionic strength, *i.e.* tissue intended for the determination of enterokinase activity should be homogenized in water or diluted buffer rather than in saline. In any case the ionic strength must be kept constant to allow a correct comparison between different samples.

The strong influence of EDTA on the enterokinase activity makes it necessary to remove this compound by dialysis before the different preparations of the brush border isolation experiment can be analyzed. If this is not done erroneous recovery and purification values may result. It should be noted that an isoionic buffer is required for a correct determination of the optimal pH for the enterokinase activity.

We feel that we have convincingly demonstrated the surprising fact that the intestinal enterokinase activity is confined to the villous epithelial cells with the subcellular localization in their brush borders. These findings suggest that the physiological activation of trypsinogen takes place at the surface membrane of the intact cells on the villi and not in the lumen as has hitherto been thought. The enterokinase activity found in the intestinal juice may then be produced by cell desquamation at the tips of the villi rather than by secretion. This kind of membrane activation would imply that there is no diffusion-barrier of importance between the lumen and the site of enterokinase function, since trypsinogen is a high molecular-weight substrate.

However, at present we do not know if the concept above is entirely correct although logical from our results. We have found[13] that the output of human enterokinase activity in the duodenal juice is rapidly and considerably increased after a test meal, and similar observations after secretin stimulation have been reported by HADORN[30]. The significance of these findings is not clear, but it is highly probable that this luminal enterokinase activity makes an important contribution to the activation of trypsinogen. The mechanism by which enterokinase is transferred from the intestinal mucosa and into the lumen is not finally explained, but long ago PAVLOV[31] stated that enterokinase is secreted from the intestinal wall on direct stimulation by pancreatic proteases. With a new approach to this subject, LEP-

KOVSKY *et al.*[32] have recently reported results which seem to confirm the old observations by PAVLOV. In experiments with depancreatized chickens they have shown that what they call "the enterokinase mechanism of the intestine", *i.e.* the level of enterokinase activity in the intestinal contents, seems to be dependent on the presence of pancreatic proteases in the lumen, and possibly exclusively trypsin. LEPKOVSKY *et al.*[32] have no aspects on their findings in relation to the localization of enterokinase in the intestinal wall, which was not known to them, but it is apparent that a direct interference between luminal factors and the enterokinase activity of the intestine may be anatomically possible owing to the superficial cellular and subcellular localization of the enzyme. It should be mentioned in this context that the enterokinase activity is easily released from isolated rat brush borders (more easily than for instance sucrase activity) during digestion with proteolytic enzymes (to be published).

What is said above does not exclude the possibility that the desquamation of cells at the tips of the villi may be the basic phenomenon responsible for the presence of enterokinase activity in the lumen. The distribution profile for enterokinase implies that this enzyme has a relatively high turnover, *i.e.* there is a considerable extrusion per unit time of cells with high enterokinase activity. In the fasting state most of this activity may remain in fragments of the cells in the superficial mucus layer covering the mucosal surface of the intestine. When the animal feeds, the pancreatic secretion may then bring about a "release".

It is of interest that enterokinase activity, like alkaline phosphatase and trehalase activities, is more apically localized on the villi than sucrase, isomaltase and maltase activities (Fig. 5, Table II). Such differences in the quantitative distribution pattern in the villi have previously been reported between alkaline phosphatase activity and maltase and sucrase activities and between alkaline phosphatase and leucyl naphthylamidase[17,33]. These differences indicate that the dynamics for different functional properties of the brush border are not identical. It is further possible that such differences are at least partly functional expressions for different changes in the ultrastructure of the brush border membrane that may occur during migration of the cells along the villi. This concept receives some support from results reported by EICHHOLZ[34]. He subfractionated the enzyme activities of the microvillus membrane from the hamster by papain digestion and found that sucrase, maltase and isomaltase activities shared a localization in the same membrane particle, which could be released from the membrane leaving behind trehalase and alkaline phosphatase activities. Similar results were obtained with isolated rat brush borders (unpublished observations by the authors).

It should be of clinical importance to note that since enterokinase is a brush-border-fixed enzyme, analysis of the enterokinase activity of mucosal biopsies must be performed on homogenates that have not been centrifuged.

When this manuscript was in final preparation we found that HOLMES AND LOBLEY[35], in a short abstract, report that they have localized the enterokinase activity to the brush-border membrane of the guinea-pig, which thus confirms our findings. No recovery values are given for the isolation experiments but it is reported that the rise in specific enterokinase activity is 31-fold in the purified brush borders and 64-fold in the brush-border membranes over the mucosal homogenate. This extremely high purification of enterokinase activity seems doubtful when compared

with the corresponding values for sucrase activity, 9-fold and 15-fold increases, respectively. Probably, such factors as the influence of EDTA and ionic strength on the assay of enterokinase activity were not considered. A further discrepancy between their results and ours is that they only find enterokinase activity in the proximal part of the small intestine, whereas we found activity along nearly the whole length of the small intestine. This may be due to different animal species used or different analytical conditions. However, considering the fundamental fact that enterokinase is a brush border enzyme our investigations agree completely.

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REFERENCES

1 B. HADORN, M. J. TARLOW, J. K. LLOYD AND O. H. WOLFF, *Lancet*, (i) (1969) 812.
2 M. J. TARLOW, B. HADORN, M. W. ARTHURTON AND J. K. LLOYD, *Arch. Disease Childhood*, 45 (1970) 651.
3 W. KÜHNE, *Virchows Arch. Pathol. Anat. Physiol.*, 39 (1867) 130.
4 R. HEIDENHAIN, *Pflueger Arch. Ges. Physiol.*, 10 (1875) 557.
5 N. P. SCHEPOWALNIKOV, *Maly's Jahresbericht über die Fortschritte der Thier-Chemie*, 29 (1900) 378.
6 I. P. PAVLOV, *The Work of the Digestive Glands*, Charles Griffin, London, 1910.
7 M. KUNITZ, *J. Gen. Physiol.*, 22 (1939) 429.
8 I. YAMASHINA, *Arkiv. Kemi*, 9 (1956) 225.
9 I. YAMASHINA, *Acta Chem. Scand.*, 10 (1956) 739.
10 E. W. DAVIE AND H. NEURATH, *J. Biol. Chem.*, 212 (1955) 515.
11 C. NORDSTRÖM AND A. DAHLQVIST, *Biochim. Biophys. Acta*, 198 (1970) 621.
12 C. NORDSTRÖM AND A. DAHLQVIST, *Symp. on Intestinal Mucosal Enzymes, 4th World Congress of Gastroenterology, Copenhagen, 1970*, Abstr. No. 130.
13 C. NORDSTRÖM AND A. DAHLQVIST, *Abstr. Annual Meeting of European Society for Pediatric Gastroenterology, Lund, 1970, Acta Paediat. Scand.*, 60 (1971) 369.
14 J. H. MILSTONE, M. V. GOLDBLAT AND V .K. MILSTONE, *Proc. Soc. Exptl. Biol. Med.*, 131 (1969) 1438.
15 H. B. HADORN, *Ph. D. Thesis*, University of Melbourne, 1969.
16 A. DAHLQVIST AND C. NORDSTRÖM, *Biochim. Biophys. Acta*, 113 (1966) 624.
17 C. NORDSTRÖM, A. DALQVIST AND L. JOSEFSSON, *J. Histochem. Cytochem.*, 15 (1967) 713.
18 C. NORDSTRÖM, O. KOLDOVSKY AND A. DAHLQVIST, *J. Histochem. Cytochem.*, 17 (1969) 341.
19 D. MILLER AND R. K. CRANE, *Anal. Biochem.*, 2 (1961) 284.
20 J. W. PORTEOUS, in G. D. BIRNIE AND S. M. FOX, *Subcellular Components*, Butterworths, London, 1969, p. 57.
21 G. G. FORSTNER, S. M. SABESIN AND K. J. ISSELBACHER, *Biochem. J.*, 106 (1968) 381.
22 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
23 M. EGGSTEIN AND F. H. KREUTZ, *Klin. Wochschr.*, 33 (1955) 879.
24 B. ERLANGER, N. KOKOWSKY AND W. COHEN, *Arch. Biochem. Biophys.*, 95 (1961) 271.
25 O. A. BESSEY, O. H. LOWRY AND M. J. BROCK, *J. Biol. Chem.*, 164 (1946) 321.
26 B. CLARK AND J. W. PORTEOUS, *Biochem. J.*, 88 (1963) 20P.
27 A. DAHLQVIST, *Anal. Biochem.*, 22 (1968) 99.
28 F. MOOG AND R. GREY, *J. Cell Biol.*, 32 (1967) C 1.
29 M. R. MCDONALD AND M. KUNITZ, *J. Gen. Physiol.*, 25 (1941) 53.
30 H. B. HADORN, *Abstr. Annual Meeting of European Society for Pediatric Gastroenterology, Lund, 1970, Acta Paediat. Scand.*, 60 (1971) 369.

31 I. P. PAVLOV, in *Les Prix Nobel en 1904*. P. A. Norstedt and Söner for the Nobel Foundation, Stockholm, 1907.
32 S. LEPKOVSKY, F. FURUTA, M. K. DIMICK AND I. YAMASHINA, *Poultry Sci.*, 49 (1970) 421.
33 R. D. GREY AND T. S. LECOUNT, *J. Histochem. Cytochem.*, 18 (1970) 416.
34 A. EICHHOLZ, *Biochim. Biophys. Acta*, 163 (1968) 101.
35 R. HOLMES AND R. W. LOBLEY, *Proc. Physiol. Soc., J. Physiol.*, 211 (1970) 50P.