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REVIEW ARTICLE

Activation of Pancreatic Zymogens
Normal Activation, Premature Intrapancreatic Activation, Protective Mechanisms Against Inappropriate Activation

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NORMAL ACTIVATION OF ZYMOGENS

The biogenesis of pancreatic digestive enzymes includes several proteolysis steps which occur sequentially during the transport of the nascent enzyme from its site of synthesis on the ribosomes to its final destination in the luminal space of the duodenum (Figure 1). The first two steps, the removal of the initiator methionine and the transport peptide, take place cotranslationally at the level of the rough endoplasmic reticulum; the final event, the removal of the inhibitor peptide or activation peptide as it is usually called, occurs in the duodenal space. This review concerns the last step only, which proceeds in two stages for all pancreatic zymogens with the exception of trypsinogen. In the first stage, active trypsin is generated from its zymogen by enterokinase, and in the second stage trypsin activates the remaining zymogens giving rise to the well-known activation cascade of pancreatic enzymes (Figure 2).

The vital role of enterokinase in normal digestion and its potential trigger action in (see below) intrapancreatic activation of zymogens in pancreatitis warrant a brief description of this enzyme. Enterokinase is synthesized by the enterocytes of the proximal small intestine. The human enzyme has been isolated and characterized only recently by Grant and Hermon-Taylor (1). It is a highly glycosylated protein with a molecular weight of 316,000 daltons, and its sole function appears to be the activation of trypsinogen. Although trypsin is also capable of activating its own zymogens, enterokinase is about 2000 times more efficient in this process than trypsin. Work by Baratti and Maroux (2) on the porcine enzyme indicates that the active site of enterokinase consists of two subsites. The first subsite is similar to that of trypsin and is responsible for the cleavage of the Lys–Ile bond in the activation of trypsinogen (see Figure 1); the second subsite, probably a cluster of lysines, interacts with the polyanionic tetraaspartyl group (Nos. 20–23, Figure 1), which is common to all mammalian trypsinogens studied so far. Binding of subsite 2 to the Asp_4 group also increases greatly the catalytic efficiency of subsite 1 and is the reason for the extraordinary specificity of enterokinase which has a single physiological substrate: trypsinogen. The enzyme is optimally active at pH 6–9, and low concentrations of bile acids enhance its activity (3, 4). It is not inhibited by biological trypsin inhibitors such as pancreatic trypsin inhibitors (Kazal or Kunitz), lima bean, soybean inhibitor, or ovomucoid, and in contrast to the pancreatic proteases, it does not form complexes with α_2-macroglobulin or α_1-antitrypsin. It is, however, inhibited by synthetic trypsin inhibitors such as aminobenzamidine, 1-chloro-3-tosylamido-7-amino heptanone (TLCK), and diisopropylfluorophosphate (DFP), and by some specific peptide aldehydes synthesized recently by Cliffe et al (5).

In view of the critical role ascribed to pancreatic enzymes in the pathogenesis of acute pancreatitis, knowledge of the normal activation process of human zymogens by human trypsins is obviously of great interest. However, no such studies have been

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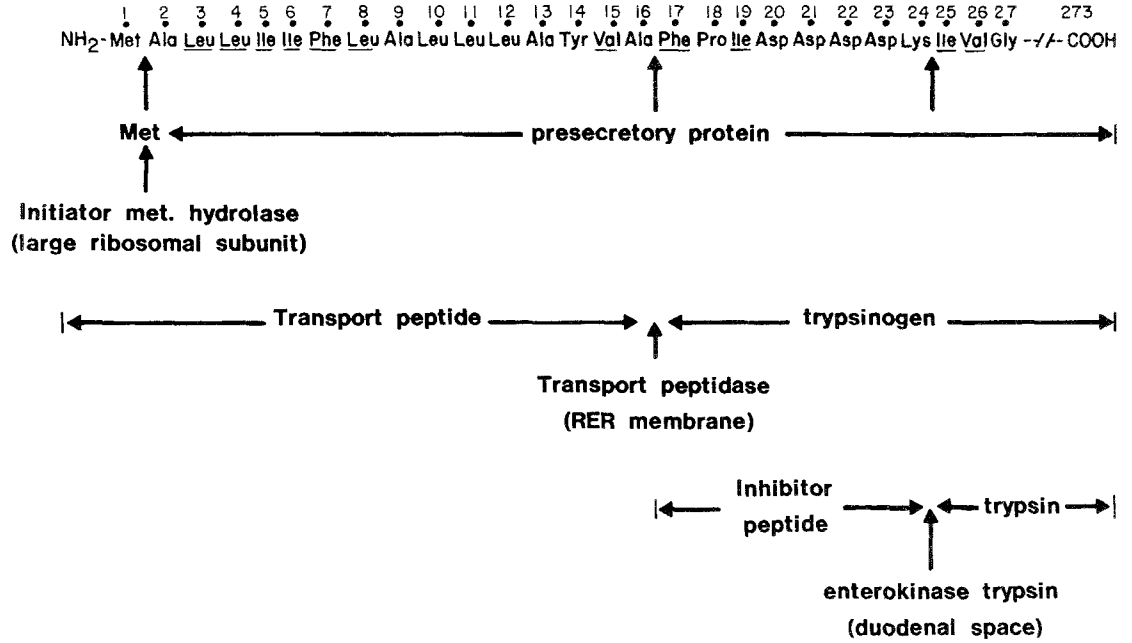

Fig 1. The biogenesis of trypsin. Amino terminal processing of canine trypsin 2. The author is indebted to Dr. G. Scheele, Rockefeller University, New York, New York, for permission to use this figure which is part of an unpublished manuscript.

reported to date, and human zymogens that have been isolated and characterized, traditionally have been activated with bovine cationic trypsin, often under nonphysiological conditions. In contrast to trypsinogen, none of the other pancreatic zymogens are capable of autoactivation and a small amount of trypsin is essential for their activation. Activation peptides split off by trypsin vary in size for different zymogens. Thus, the activation peptide of human chymotrypsinogen has 14, that of proelastase 11, that of prophospholipase A_2 7, and those of procarboxypeptidases A and B more than 100 amino acid residues. Not all of the activation peptides are severed completely from the zymogens and some of them remain attached to the active enzyme by a disulfide bridge. Such is the case for human chymotrypsin and elastase.

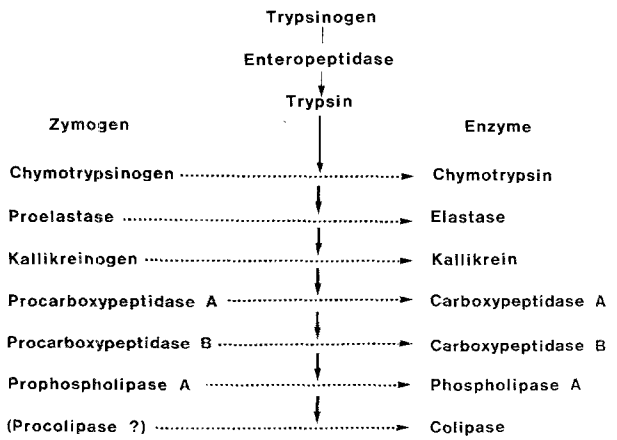

Fig 2. The pancreatic enzyme cascade.

Activation of zymogens in the small intestine probably is more complex than indicated in Figure 2, and autocatalytic or secondary cleavage of the initial enzymes may lead to less active species, as has been observed for bovine trypsin and especially for bovine chymotrypsin which is degraded autocatalytically to the well characterized α-, β-, γ-, and δ-chymotrypsins (6). The proportions of active and partially degraded enzymes are determined by a variety of factors, including the concentrations of zymogen, active trypsin, and total protein; pH; temperature; and length of incubation. The presence of Ca generally has a stabilizing effect on the enzymes. No analogous studies of the corresponding human enzymes have been reported to date, but recent observations point to the existence of degradation products of human cationic trypsin which may have physiological or pathophysiological significance (see below and ref. 7). Furthermore, it has been shown that the rate of activation of human zymogens often differs considerably from that of animal zymogens. Thus, human cationic trypsinogen autoactivates far more readily than bovine cationic trypsinogen, and human prophospholipase A_2 according to Figarella et al (8) is considerably

more resistant to activation than the porcine zymogen. The ease with which specific zymogens, particularly proelastase and prophospholipase A_2 (9), are activated undoubtedly plays a critical role in the pathogenesis of acute pancreatitis, and systematic studies of the activation of human zymogens by human trypsins should furnish much needed information.

INAPPROPRIATE INTRAPANCREATIC ACTIVATION OF ZYMOGENS

Although it is virtually certain that pancreatic enzymes are involved in the autodigestion of the pancreas in acute necrotizing (hemorrhagic) pancreatitis, the enigma of how trypsin, the trigger enzyme in this process, is activated has remained unexplained to date. There is, however, no dearth of hypotheses attempting to explain this mechanism, most of them based on *in vitro* evidence or on observations made in animal models. Four of them will be considered here, but the possibility should be kept in mind that there may be more than one *in vivo* mechanism capable of activating the trigger enzyme of the pancreatic enzyme cascade.

Autoactivation. Sarles (10) and coworkers have shown that formation of protein precipitates in pancreatic ducts plays an important role in the pathogenesis of chronic calcifying pancreatitis. Recently, Figarella et al (7) have presented evidence that protein precipitates (pancreatic stone protein) in pancreatic juice of such patients may be derived from cationic trypsinogen. This observation, together with the frequent finding of small amounts of free proteolytic activity in pancreatic secretions of such patients, suggests intrapancreatic autoactivation of trypsinogen. Indeed, Kassell and Kay (11) showed, many years ago, that bovine trypsinogen possesses inherent proteolytic activity and is capable of self-activation, and Colomb and coworkers (12) more recently have demonstrated that human trypsinogens can bind substrates, pseudosubstrates, and trypsin inhibitors, and are, especially the cationic form, even more susceptible to autoactivation than the bovine zymogen. However, conditions in pancreatic juice are far more favorable to degradation than activation of trypsinogen (see below), and it appears unlikely that activation of the pancreatic enzyme cascade in acute pancreatitis is initiated by this mechanism.

Activation by Plasmin (Thrombin). It is well known that activators of plasminogen producing plasmin are released by a variety of factors such as bacterial toxins, elctroshock, ischemia, anoxia, etc. Some of these conditions are associated with the release of lysosomal hydrolases and other products from polymorphonuclear leukocytes. It has been postulated that increased capillary permeability, a concomitant of this event, could allow transudation of proteases of the clotting and clot-lysing systems through the periacinar membrane leading to activation of trypsinogen by plasmin (or thrombin) and ignition of the process of autodigestion. Activation of bovine trypsinogen by plasmin has been demonstrated *in vitro* (13), but more extensive evidence for this trigger mechanism in human pancreatitis has not been obtained to date. Thrombin also has been reported to activate trypsinogen (14), but we have been unable to duplicate this experiment in our laboratories. However, it is interesting to mention here that thrombin, according to observations by Van Dam-Mieras et al (in ref. 15), is capable of activating the zymogen of human and equine phospholipase but not that of other species. The significance of this important finding in the pathogenesis of human pancreatitis (9) has not been evaluated so far.

Activation of Trypsinogen by Lysosomal Cathepsin B. Recent work by Koike et al (16) with mice fed a choline-deficient diet supplemented with ethionine suggests that the hemorrhagic pancreatitis resulting from this diet essentially is a consequence of blocked zymogen discharge from the acinar cell in the face of continued zymogen synthesis and intracellular transport. Accumulation of zymogen granules in the acinar cell cannot continue indefinitely and induces crinophagy. This is the normal process by which secretory cells divest themselves of excess stored secretory products. It involves discharge of these products, in this case the contents of zymogen granules, into lysosomal compartments by fusion of the corresponding limiting membranes to form a crinophagic vacuole. One of the lysosomal enzymes known to occur in human pancreatic tissue is cathepsin B. *In vitro* experiments have shown that this hydrolase is capable of activating human trypsinogen at pH 3.8 (17). Its presence in a crinophagic vacuole obviously presents a potential danger. If crinophagy is massive, the capacity of acinar cells to keep it at a physiological level might be overwhelmed, and crinophagic vacuoles could rupture and spill their contents into the interstitium. The ensuing increase in pH would then permit activation of pancreatic zymogens by trypsin lead-

ing to autodigestion of the pancreas. Since small amounts of active proteases have indeed been found by Rao et al (18) in the pancreas of experimental animals during feeding with this diet, it was postulated by Koike et al (16) that crinophagic vacuoles may be the site of activation of trypsinogen by lysosomal cathepsin B.

In another model of experimental pancreatitis in rats, Lampel and Kern (19) have shown that supramaximal stimulation of animals with secretagogues, such as cerulein, leads to the formation of large vacuoles containing zymogens as well as lysosomal enzymes in the cytoplasm of acinar cells and to abnormal, basolateral exocytosis of zymogen granules into the interstitial spaces. Although experimental evidence is as yet lacking, it is possible that, here too, cathepsin B may be involved in activation of trypsinogen and the induction of pancreatitis. These animal models present intriguing new evidence or at least insight into possible trigger mechanisms of pancreatic autodigestion, but their relevance to the human disease remains to be investigated in future studies.

Activation of Trypsinogen by Enterokinase. Inappropriate sequestration of enterokinase into the pancreas was suggested by McCutcheon (20) as a cause of pancreatitis almost 20 years ago. However, reflux of bile and active proteases from the duodenum into the pancreas normally is prevented by the ampullary sphincter and is rarely seen during radiological studies even after sphincterotomy or papillotomy. Recently, a group of British investigators has taken a second look at the potential role of enterokinase in the premature intrapancreatic activation of zymogens. Talbot et al (21) observed that gentle, intermittent perfusion of guinea pig duodenum with water or saline displaced some enterokinase into the portal venous blood. This suggests that absorption of enterokinase into the portal circulation may be a physiological event and likely to occur after a meal. Enterokinase was not detectable in the blood of nonperfused control animals. The half-life of the enzyme in the circulation is only about 2.5 min due to its rapid clearance by hepatocytes which possess receptors that recognize enterokinase by a portion of its carbohydrate mantle. Although 98–99% of the enzyme is destroyed by lysosomal hydrolases in endocytotic vesicles of hepatocytes, 0.2–2% of the active enzyme is secreted into the bile within 1 hr. It is interesting to note that impairment of hepatocyte function, as in ethanol-induced fatty liver in rats, increases enterokinase concentration in bile two- to threefold.

In contrast to duodenal–pancreatic reflux, biliary–pancreatic reflux is seen frequently during routine biliary radiology, and the above findings suggest that bile-borne enterokinase entering the pancreatic duct system may, in some circumstances, be implicated in setting off the process of pancreatic autodigestion by activating trypsin, the trigger enzyme of the pancreatic enzyme cascade. Indeed, Terry et al (22) have shown that 50–200 ng enterokinase in 0.1 ml 30 mM glycodeoxycholate solution, when infused into the pancreatic duct of rats, causes fatal hemorrhagic pancreatitis. Furthermore, the same group of workers detected enterokinase in postoperative bile of 14 patients with biliary disease, but no demonstrable duodenal reflux (23). Concentrations of enterokinase in bile collected over a 4-hr period varied from 0.1 to 4.8 ng/ml. The presence of this enzyme in bile probably reflects changes in liver function following general anesthesia and surgical trauma. It may be significant that the patient with the highest concentration of biliary enterokinase (4.8 ng/ml) developed postoperative necrotizing pancreatitis. These observations, made in animal models as well as patients, suggest that short-term peak levels of enterokinase, much higher than those recorded by Grant et al (23), may be able to trigger the pancreatic enzyme cascade during reflux into the pancreas and lead to acute hemorrhagic pancreatitis.

Some observations made in our own laboratories some five or six years ago appear to support this concept. Human enterokinase, when added to fresh, unactivated pancreatic juice at 3 ng/ml, a concentration similar to that found in bile of patients with biliary disease (above), merely led to slow degradation of zymogens and negligible activation of enzymes. Trypsin inhibitor was not measurably affected (Figure 3). However, when the concentration of enterokinase was increased about 10-fold to 32 ng/ml, activation of enzymes was rapid and massive, and trypsin inhibitor disappeared almost immediately (Figure 4).

PROTECTIVE MECHANISMS AGAINST INTRAPANCREATIC ACTIVATION OF ZYMOGENS

One such mechanism has been mentioned in the introduction and is represented by the fact that

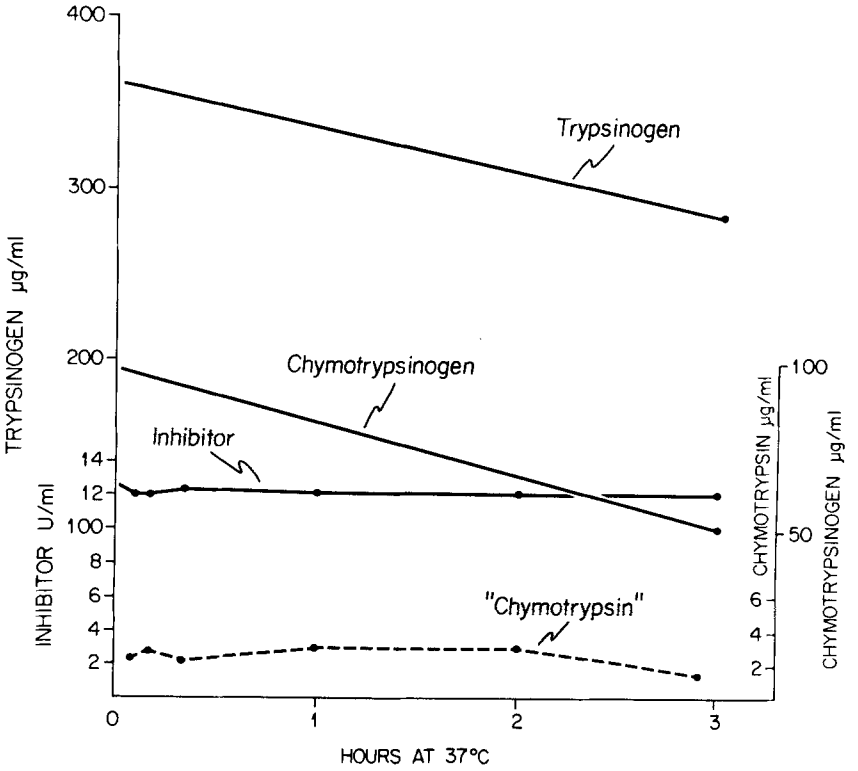

Fig 3. The action of enterokinase on fresh human pancreatic juice. Fresh human pancreatic juice, free of proteolytic activity was incubated at 37° C with human enterokinase (3 ng/ml, ref. 28). Samples were withdrawn at intervals, free proteolytic activity and trypsin inhibitor determined as indicated in ref. 29, and trypsinogen and chymotrypsinogen measured as free enzymes after activation with human enterokinase (29).

potentially dangerous enzymes are secreted in the form of inactive zymogens which normally are activated in the duodenum.

A second barrier against intrapancreatic activation of zymogens is formed by the pancreatic trypsin inhibitor (Kazal inhibitor) which is secreted in pancreatic juice along with zymogens and enzymes. This small protein (mol wt about 6000 daltons) occurs at a concentration sufficient to inhibit up to 20% of potentially available trypsin. It rapidly forms a complex with trypsin accidentally liberated in the pancreas and thus prevents activation of the pancreatic enzyme cascade. However, the complex has a relatively high dissociation constant, and a small amount of trypsin activity can still be detected by a sensitive assay even in the presence of a large excess of inhibitor. This may be important because traces of dissociated free trypsin may conceivably activate particularly susceptible zymogens such as kallikreinogen (24), chymotrypsinogen, and perhaps others. It may also explain our recent observation that the trypsin–trypsin inhibitor complex is capable of clotting fibrinogen whereas trypsin does not. Moreover, it may shed some light on the notorious instability of pancreatic juice once contaminated by a trace of trypsin.

A further obstacle to intrapancreatic activation of zymogens are the unfavorable conditions in pancreatic secretions for activation of trypsinogen by traces of trypsin, ie, the high pH (8–9.5) and the low Ca concentration (usually less than 1 mM), both of which greatly favor degradation rather than activation of trypsinogen.

Recently, we obtained evidence for an additional mechanism for the protection of the pancreas against digestion by its own enzymes. Normal human pancreatic juice contains a minor trypsinogen variant which furnishes an active enzyme (mesotrypsin) almost totally resistant to biological trypsin inhibitors, including Kazal pancreatic trypsin inhibitor (25). It seems likely that this zymogen, once activated intrapancreatically, would be capable of

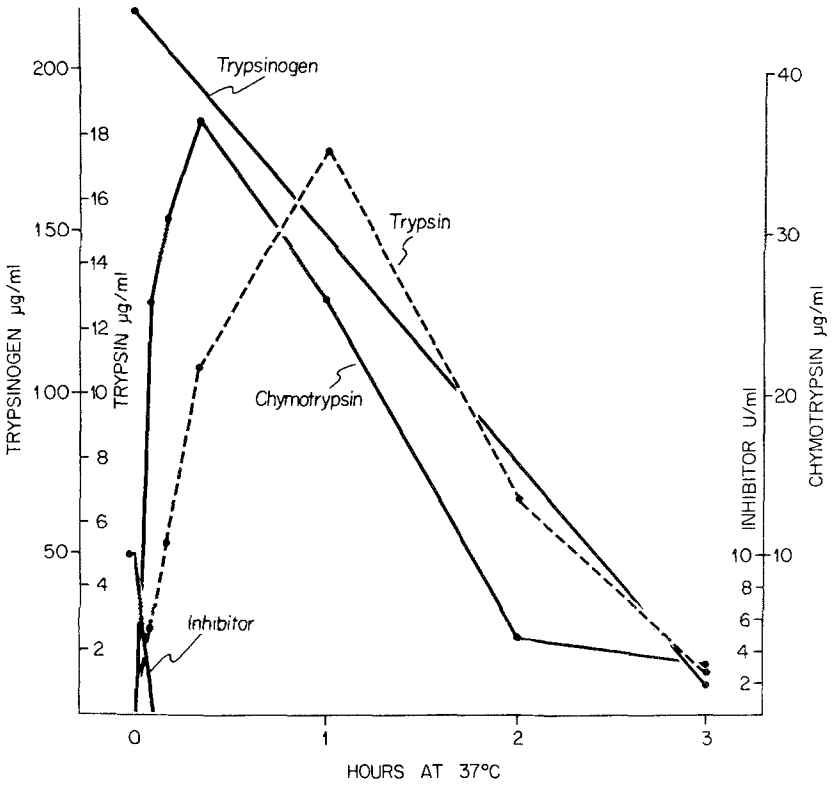

Fig 4. The action of enterokinase on fresh human pancreatic juice. Incubation of pancreatic juice with human enterokinase (32 ng/ml). Procedures as in Figure 3.

activating, unopposed even by excess trypsin inhibitor, the remaining trypsinogens and trigger the pancreatic enzyme cascade causing acute necrotizing pancreatitis.

Contrary to expectations, we found that addition of small amounts of this inhibitor-resistant enzyme to fresh, unactivated pancreatic juice (600 ng/ml) resulted in rapid degradation of the zymogens of trypsin, chymotrypsin, and elastase rather than activation to the corresponding enzymes (Figure 5). This observation favored a protective, rather than a pathogenetic, role for mesotrypsin in that it removed the potentially dangerous zymogens from the secretions rather than activating them to the corresponding enzymes. However, our efforts to clarify these findings suggested a much more complicated mechanism than indicated by the above experiments: Incubation of a semipurified fraction of human cationic trypsinogen, obtained by gel electrophoresis (26), with highly purified mesotrypsin (25) again led to complete degradation of trypsinogen within 1 hr, but unexpectedly, so did the control experiment in which no mesotrypsin had been added (left side of Figure 6). The cationic trypsinogen fraction used in this experiment was free of all but traces of other zymogens, showed only barely detectable traces of trypsin-like activity, but was still contaminated with pancreatic trypsin inhibitor. The incubate obtained in 1 hr at 37° C, designated as enzyme Y for brevity's sake, showed only questionable activity when tested for trypsin, chymotrypsin, elastase, and other proteases, but trypsin inhibitor activity was not diminished. However, when a portion of enzyme Y was added to a solution of anionic trypsinogen (obtained by gel electrophoresis as above) with a Ca content of <1 mM, and totally free of trypsin activity, trypsinogen was degraded almost completely within 60 min at 37° C (right side of Figure 6). Figure 6 also shows that treatment of enzyme Y with the specific trypsin inhibitor 1-chloro-3-tosylamido-7-amino heptanone (TLCK) did not affect its activity, whereas incubation with diisopropylfluorophosphate (DFP) totally abolished it.

The results of a large number of similar probes, summarized in Table 1, led us to the tentative conclusion that we were dealing here with a hitherto unrecognized pancreatic protease or zymogen acti-

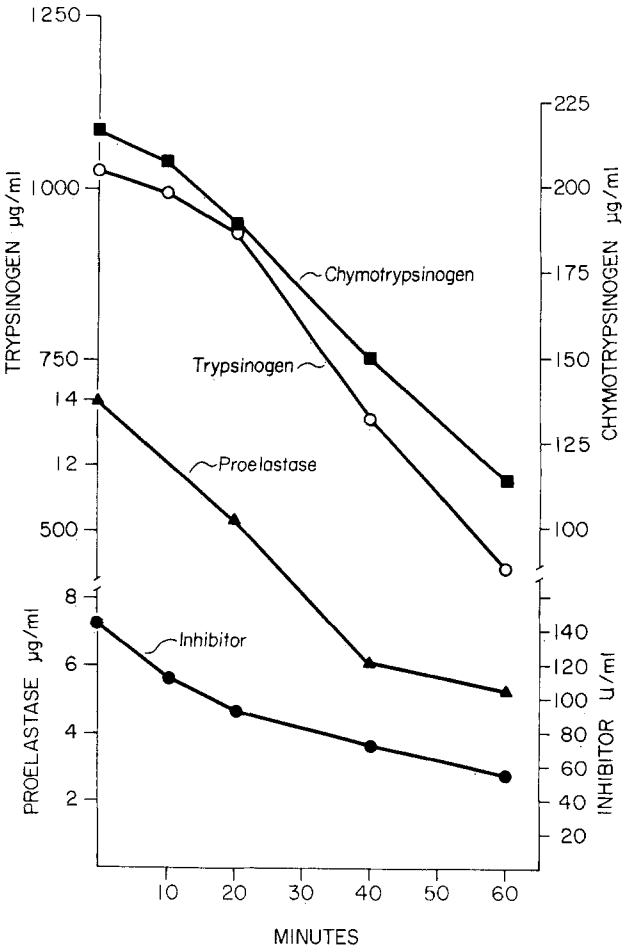

Fig 5. Incubation at 37° C of fresh human pancreatic juice with purified mesotrypsin (25) (600 ng/ml). Samples were withdrawn at intervals indicated and enzymes, inhibitor, and zymogens determined as outlined in Figure 3.

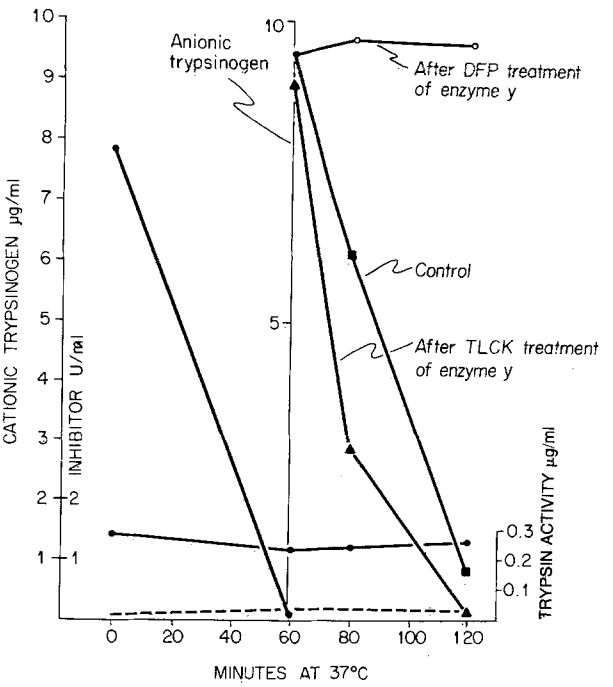

Fig 6. Degradation of trypsinogen by enzyme Y. Left side: Degradation of human cationic trypsinogen obtained by gel electrophoresis (26). Cationic trypsinogen contained only traces of other zymogens, was free of mesotrypsinogen, but still contaminated by pancreatic secretory trypsin inhibitor. It also showed a trace of questionable trypsin-like activity. Samples were withdrawn during incubation at 37° C as indicated and enzymes, zymogen, and inhibitor determined as shown in Figure 3. Right side: ■—■, degradation of anionic trypsinogen by enzyme Y (= cationic trypsinogen after incubation at 37° C for 60 min, see text). Anionic trypsinogen was obtained by gel electrophoresis of human pancreatic juice (26), showed no detectable trypsin activity, and was free of other protease zymogens and inhibitor. Ca concentration was titrated to less than 1 mM with an EDTA solution. ▲—▲, incubation of anionic trypsinogen with enzyme Y previously treated with TLCK. ○—○, incubation of anionic trypsinogen with enzyme Y previously treated with DFP. Incubation and determination of enzyme, zymogen, and inhibitor as in Figure 3.

vated during incubation, possibly by traces of trypsin dissociating from a small amount of trypsin–trypsin inhibitor complex generated during electrophoresis of pancreatic juice. Alternatively, enzyme Y may be a product of autodigestion of cationic trypsinogen with a greatly altered enzymatic specificity. A precedent for this concept is the pseudotrypsin described as an autolysis product of bovine trypsin by Smith and Shaw (27). Like enzyme Y, it possesses greatly reduced activity towards *N*-benzoyl-arginine *p*-nitroanilide, is not inhibited by TLCK, but is inactivated by diisopropylfluorophosphate. Further work is needed to characterize the activity responsible for the destruction of zymogens observed in these experiments, but the results presented here show, I think unequivocally, the existence of a hitherto unrecognized intrapancreatic mechanism for the rapid removal of zymogens from an environment likely to trigger the pancreatic enzyme cascade.

TABLE 1. PROPERTIES OF ENZYME Y

1. It is a serine protease (inhibited by DFP)
2. It has little or no activity towards a variety of synthetic protease substrates, but degrades human trypsinogens
3. Activity is high at pH 8.3, low at pH 5.4
4. Ca^{2+} > 1 mM is inhibitory to enzyme activity
5. Activity is not inhibited by TLCK, TPCK, Trasylol, Hg^{2+}, or iodoacetate, but is reduced by increasing concentrations of pancreatic secretory trypsin inhibitor
6. It is stable at pH 1.9 and 37°C for 30 min
7. It is probably derived from a zymogen in unactivated pancreatic juice
8. These properties preclude its identity with that of any of the known pancreatic proteases

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