22. In this connection, it is interesting to note that etic concepts for the classification of alphabetic and syllabic systems have received far more attention—and have proven far more adequate—than those used to classify simpler forms of writing. See, for example, the classification of alphabetic systems by C. F. Voegelin and F. M. Voegelin (*6*).

23. We thank the following Western Apaches, whose understanding, cooperation, and friendship made this study possible: Silas John Edwards, for permission to undertake the study, to publish the results, and for providing us with essential information about the origin and history of his writing system; Marshall Miller, for gifted instruction in how to read; John Nolene, for his generous loan; Marion and Violet Zahgotah, who allowed us to disrupt the daily routine of their household and never once complained; and Maggie Anderson, who opened her home to us as a base of operations and whose advice, warmth, and sheer goodwill made everything that much better. We also thank David Schneider who brought the Silas John script to our attention by pointing out the presence of Harry Hoijer's Mescalero texts in an unpublished manuscript by the late Jules Henry. Henry had given Schneider access to the manuscript, and Schneider supposed, quite correctly, that it would be of singular interest to us. We are grateful to Harry Hoijer for allowing us to examine his original field notes. Finally, we thank Bernard Fontana, Dell Hymes, William Sturtevant, and Sol Tax, who urged us not to delay our investigation and took steps that enabled us to begin. Our field research was sponsored by the Center for the Study of Man, Smithsonian Institution, Washington, D.C., and the Doris Duke American Indian Oral History Project, Arizona State Museum, University of Arizona. Finally, we thank the following scholars, whose comments on an earlier draft of this paper were especially helpful: Ellen Basso, Roy D'Andrade, Harold Conklin, Regna Darnell, Bernard L. Fontana, I. J. Gelb, Erving Goffman, McGuire Gibson, Philip Greenfeld, Kenneth Hale, Harry Hoijer, Dell Hymes, Morris Opler, Alfonso Ortiz, Joel Sherzer, William Sturtevant, and Richard Thompson.

Zymogens of Proteolytic Enzymes

These enzyme precursors, formerly thought to be inert substances, have inherent proteolytic activity.

Beatrice Kassell and John Kay

The enzymes that digest proteins in the alimentary canal are synthesized as precursors called zymogens. These zymogens are stored in granules (*1*) in the synthesizing organ; for example, pepsinogen in the lining of the stomach and trypsinogen, chymotrypsinogen, proelastase, and procarboxypeptidases in the pancreas. Synthesis as precursors and storage in granules protect the tissues from self-destruction by their own enzymes.

The ingestion of food causes secretion of hydrochloric acid into the stomach along with pepsinogen and other components of the gastric juice. On contact with acid, by a process called autoactivation, pepsinogen is converted to pepsin (*2*), which is the enzyme responsible for gastric digestion of proteins. Similarly, the exocrine pancreas secretes its zymogens into the duodenum, the first part of the intestine, where the activation process begins with the conversion of trypsinogen to trypsin. This activation can be accomplished in two ways, either by the action of enterokinase, an enzyme of the intestinal wall (*3*), or by autoactivation. Under physiological conditions, activation by enterokinase is probably the predominant reaction (*4*) but in vitro, trypsinogen undergoes autoactivation in the absence of enterokinase (*3*).

Trypsin is a key enzyme important for the conversion of other zymogens to their active enzymes, for example, chymotrypsinogens to chymotrypsins, proelastase to elastase, and procarboxypeptidases to carboxypeptidases. These enzymes, all acting at their own specific sites in protein macromolecules, cooperatively break down food proteins to small peptides and amino acids.

The activation of pepsinogen and trypsinogen in vitro are the two classical examples of autoactivation (*2, 3*). Activation begins slowly, but as enzyme is formed, it catalyzes further activation and a rapid acceleration takes place. An S-shaped curve is obtained for the appearance of enzymic activity, as illustrated for trypsinogen in Fig. 1. It has been assumed for many years that activation is initiated by a small amount of active enzyme present as a contaminant. This does not explain how the *first* molecule of pepsin or trypsin was formed.

The process of conversion of the zymogens to enzymes occurs by hydrolysis of a peptide linkage, usually accompanied by removal of a fragment from the amino terminal of the protein chain, and thus involves proteolytic action by the activating agent. The formation of physiologically active molecules by limited proteolysis has been reviewed (*5*) and is not restricted to formation of digestive enzymes or to higher organisms. To cite a few examples, insulin is formed from a larger protein called proinsulin (*6*); a series of proteolytic reactions forms the enzymes that participate in blood clotting (*7*); phospholipase A of porcine pancreas is formed from its zymogen by the action of trypsin (*8*); the peptide hormone, gastrin, is derived from a larger molecule (*9*); and a streptococcal proteinase is derived from a zymogen (*10, 11*). Thus these zymogen to enzyme conversions are part of a general process by which macromolecules are synthesized in precursor forms and are converted by enzymic modification to physiologically active substances.

In this article we discuss the recent discovery that certain zymogens have inherent enzymic activity so that in some instances they are capable of activating themselves. In this way, they are capable of producing the elusive first enzyme molecule. We consider the evidence for this activity in some detail, using as illustrations the zymogens of different types of proteolytic enzymes: acid proteases, serine proteases, a sulfhydryl enzyme, and a metalloenzyme.

Acid Proteases

The first suggestion that a zymogen may have activation power of its own was made by Foltmann (*12, 13*) who conducted studies with prorennin, the precursor of the milk-clotting enzyme, rennin (chymosin) of the calf stomach. Above *p*H 5.3 prorennin is relatively stable. Between *p*H 5 and *p*H 2, as the *p*H is lowered the rate of activation is

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accelerated. Based on deviation of the observed rate of autoactivation from the predicted autocatalytic reaction (Fig. 2) Foltmann suggested a new hypothesis for the activation. He assumed that the inactive prorennin molecule is stabilized by the amino terminal portion of the peptide chain, which is later split off during the activation, and that this portion of the molecule is kept in place by electrostatic interaction between positively and negatively charged groups. When the *p*H is decreased, the carboxyl groups become protonated, thereby weakening the electrostatic forces. Under such conditions, some of the prorennin molecules may rearrange into an active conformation and thus be capable, without having undergone limited proteolysis themselves, of activating other prorennin molecules. The lower the *p*H value, the more the reaction deviates from the curve of an autocatalytic reaction; at *p*H 2, the rate of activation, in fact, resembles a second order reaction.

Support for the idea of stabilization of the zymogen by binding of the amino terminal segment with another portion of the molecule is provided by the experiments of Wong *et al.* (*14*) with the related zymogen from the adult cow, pepsinogen. Two lysine residues of pepsinogen, one from the amino terminal region and one from the carboxyl terminal region, were cross-linked chemically by reaction with gossypol, a dialdehyde isolated from cotton seeds. Thus, these two lysine residues must lie close together in the pepsinogen molecule. The cross-linked pepsinogen could not be activated (*15*).

Pepsinogen is stable in neutral solution. Below *p*H 5 it is converted to pepsin by cleavage of several peptides from its amino terminal (*16*); the rate of conversion is dependent on *p*H. Bustin *et al.* (*11*) were the first to suggest the possibility that an active species of pepsinogen might exist prior to this proteolysis. Since activation occurs only in acid solution and activity can be measured only under the same conditions, it is obviously difficult to determine whether pepsinogen has inherent proteolytic activity and whether pepsinogen or pepsin is present at any moment. Special conditions to measure the activity of pepsinogen without interference by pepsin were devised by Bustin and Conway-Jacobs (*17*); we have selected one of their experiments as an illustration.

The basis of the experiment was the presence of a large excess of a protein substrate in a solution containing only a very low concentration of pepsinogen, thus making it unlikely either that pepsinogen molecules would come into contact with each other, or that a pepsin molecule from a trace of contaminating pepsin would come in contact with a pepsinogen molecule. A partially digested hemoglobin substrate was present at a concentration of 12.5 milligrams per milliliter while the pepsinogen concentration was only 8.4 micrograms per milliliter. Digestion was measured by formation of trichloroacetic acid–soluble products, determined by their absorbance at 280 nanometers. Figure 3 shows that the amount of digestion of the substrate by 8.4 μg of pepsinogen per milliliter was approximately equal to that produced by 2.1 μg of pepsin per milliliter (curves A and B), whereas 8.4 μg of pepsin per milliliter gave a rate four times as high (curve D). The rate was additive when pepsinogen and pepsin were both added (separately) to the same substrate solution (curve C); that is, the presence of pepsin, under these conditions, did not affect the rate at which pepsinogen acted on the substrate. The authors concluded that either the zymogen, pepsinogen, is able to digest a protein substrate, or it is able to convert itself to pepsin in a monomolecular reaction.

Al-Janabi *et al.* (*18*) subsequently deduced from kinetic measurements that the type of reaction varies with the *p*H, the reaction being predominantly intramolecular below *p*H 3, this confirming Bustin and Conway-Jacob's proposal, but at *p*H 4 the reaction is predominantly intermolecular. Funatsu *et al.* are also in favor of an intramolecular mechanism, their evidence coming from studies (*19, 20*) of activation in the presence of a competitive inhibitor or of a large excess of a synthetic substrate. McPhie (*21*) has shown that kinetic measurements and calculations indicate that care must be exercised before one decides whether the activation mechanism is mono- or bimolecular.

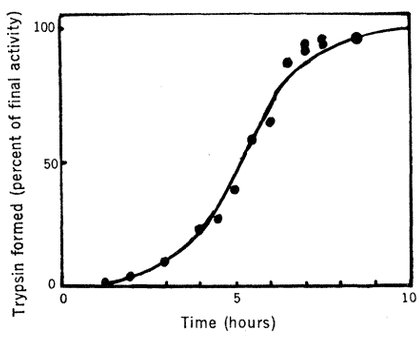

Fig. 1. Autocatalytic activation of trypsinogen. The curve is calculated from the equation for a simple autocatalytic reaction; solid circles show the observed activity. [After Kunitz and Northrop (*3*)] [Permission of the Rockefeller University Press]

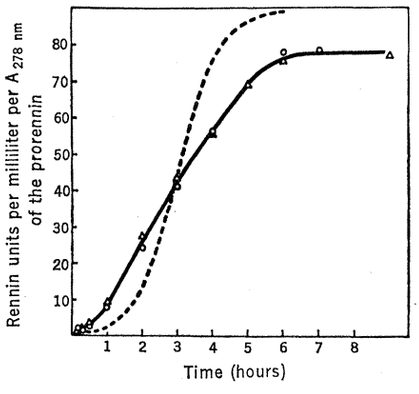

Fig. 2 (left). Activation of prorennin at *p*H 4.7 and 25°C. The concentration of prorennin was 0.95 mg/ml. The results show the ratio of the milk-clotting activity in rennin units per milliliter to the absorbancy of the prorennin solution at 278 nm; broken line indicates the course of an autocatalytic reaction. [From Foltmann (*12, 13*)] [Permission of Academic Press]

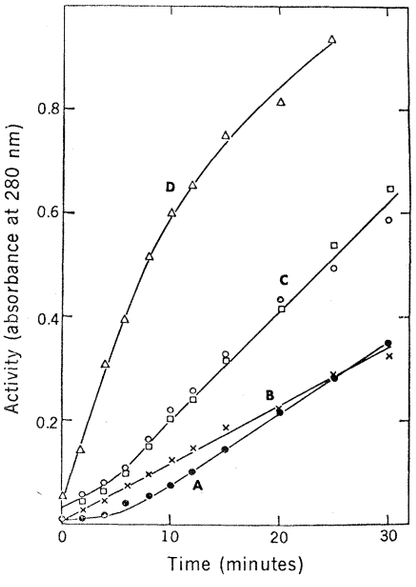

Fig. 3 (right). Digestion of partially digested hemoglobin at *p*H 3.0 and 23°C by solutions of pepsin, pepsinogen, and a mixture of both: curve A, 8.4 μg of pepsinogen per milliliter; curve B, 2.1 μg of pepsin milliliter; curve D, 8.3 μg of pepsin per milliliter; curve C, mixture of 2.1 μg of pepsin per milliliter plus 8.4 μg of pepsinogen per milliliter: squares, experimental results; open circles, calculated from the sum of pepsinogen by itself and pepsin by itself. [From Bustin and Conway-Jacobs (*17*)] [Permission of the American Society of Biological Chemists]

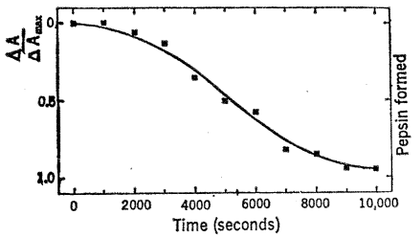

Fig. 4. Kinetics of the absorbance change ($\Delta A/\Delta A_{max}$) at 287.5 nm after the acidification of a pepsinogen solution from 0.1*M* sodium chloride, 2 m*M* tris, *p*H 7.5, by the addition of sodium formate buffer to a final concentration of 10 m*M*, *p*H 4.5. Solid squares show the appearance of peptic activity in the same solution. [From McPhie (*21*)] [Permission of the American Society of Biological Chemists]

Table 1. Activation of trypsinogen in the presence and absence of soybean trypsin inhibitor. At each concentration of zymogen, incubation with or without inhibitor (molar ratio of zymogen to inhibitor of 300 : 1) was conducted at 32°C in 0.1*M* tris(hydroxymethyl)-aminomethane (tris), *p*H 8.1, containing 0.05*M* Ca^{2+}. Lag time is defined as the time for the first appearance of detectable tryptic activity. [From Kay and Kassell (*27*)] [Permission of the American Society of Biological Chemists]

Trypsinogen (mg/ml)	Lag time (minutes)	
	With inhibitor	Without inhibitor
7.15		30
2.0		45
1.0	200	90
0.4	950	600
0.1	6500	3600

The conformational change that occurs when pepsinogen is exposed to acid solution (*19, 21, 22*) led to the suggestion that the transformation produces an active species of pepsinogen which then catalyzes cleavage of the peptides. This is analogous to Foltmann's hypothesis for prorennin activation. McPhie (*21*) demonstrated a parallel change in spectral properties and appearance of activity (Fig. 4). He then did a very rapid measurement of the spectral change at *p*H 2.85, and neutralized the solution within 2 minutes. It is well known that activity appears within this time (see Fig. 3); yet the zymogen was restored to a form having the spectrum of native pepsinogen (Fig. 5). Thus, the appearance of activity cannot at this stage involve a chemical change such as cleavage of peptides to form pepsin.

There is agreement among the various groups studying prorennin and pepsinogen that active zymogens are formed which somehow produce the active enzymes. There is not agreement, however, on whether this transformation occurs in a monomolecular or a bimolecular reaction. It appears to us that, although the conformational change is necessarily a monomolecular reaction, the second step of conversion of the active zymogen to enzyme is more likely to be bimolecular, that is, an "active zymogen" molecule cleaving a peptide bond in another zymogen molecule. Under the conditions of the pepsinogen experiment in which a large excess of hemoglobin substrate was present (Fig. 3), the second step would not occur at all. If gradual conversion to pepsin were taking place, the rate of digestion of hemoglobin by pepsinogen (Fig. 3, curve A) would not be linear and would not be one-fourth of the rate that occurs with pepsin (curve D). Instead the rate would be exponential, approaching the rate of digestion by pepsin. Considered in this way, all of the data can be reconciled.

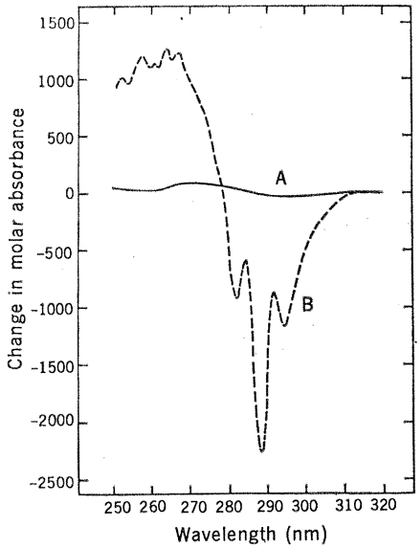

Fig. 5. Difference spectra of pepsinogen solutions. Curve A, 20 μM pepsinogen, taken to *p*H 2.85 and reneutralized within 2 minutes; reference, 20 μM pepsinogen, *p*H 7.5. Curve B, 20 μM pepsinogen, taken to *p*H 2.85 and reneutralized after 75 minutes; reference, 20 μM pepsinogen, *p*H 7.5. [From McPhie (*21*)] [Permission of the American Society of Biological Chemists]

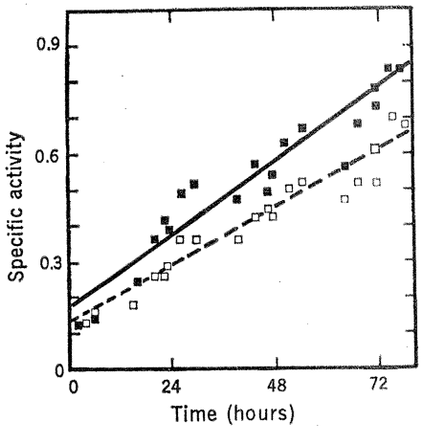

Fig. 6. Activation of chymotrypsinogen A by acetyltrypsinogen. Acetyltrypsinogen (5.5 mg) in 3.0 ml of 0.1*M* tris-HCl, *p*H 8.1, containing 0.05*M* $CaCl_2$, was incubated with soybean inhibitor (19 μg). After 5 minutes at 30°C, chymotrypsinogen (12 mg) was added, and incubation was continued. Samples were removed for detection of chymotryptic activity with benzoyltyrosine ethyl ester as substrate. Solid squares, acetyltrypsinogen previously treated with DFP; open squares, acetyltrypsinogen not treated with DFP. [From Kay and Kassell (*27*)] [Permission of the American Society of Biological Chemists]

"Serine" Proteases

Trypsin is an example of a large class of enzymes with a reactive serine at the catalytic site. Trypsin is most active in the *p*H range 7 to 9. Calcium ion increases the stability and the activity of trypsin solutions and is a necessary component for the complete conversion of trypsinogen to trypsin by autocatalytic activation (*3*).

The conversion of bovine trypsinogen into trypsin is effected by release of a single peptide, Val-Asp-Asp-Asp-Asp-Lys (*23*), from the amino terminal of the zymogen (*24*), accompanied by a conformational change (*25*). Trypsinogen is stable at *p*H 3, but when it is dissolved in neutral or slightly alkaline solution, conversion to trypsin occurs (*3*). The rate of this activation increases rapidly as the trypsin formed begins to catalyze the activation reaction (Fig. 1).

To determine whether trypsinogen has inherent proteolytic activity that enables it to activate itself, we designed two kinds of experiments to exclude the effect of possible contamination by trypsin (*26, 27*).

In the first set of experiments, the activation of trypsinogen was measured in the presence of two kinds of inhibitors: soybean trypsin inhibitor (*3*), a protein inhibitor that binds trypsin with an inhibition constant of $10^{-10}M$ (*28*) or diisopropyl fluorophosphate (DFP), an inhibitor that reacts specifically and irreversibly with the serine residue at the active site of trypsin (*29*).

Both inhibitors, added in amounts more than sufficient to inactivate any trace of trypsin in the trypsinogen,

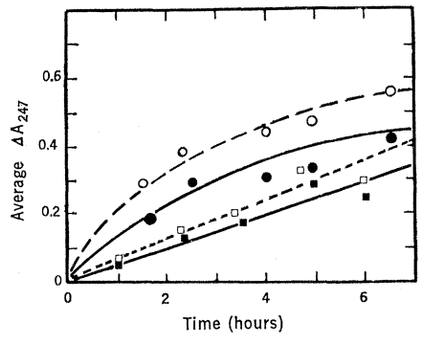

Fig. 7. Hydrolysis of tosylarginine methyl ester (TAME) by acetyltrypsinogen. Acetyltrypsinogen (open and solid circles, 1 mg/ml; open and solid squares, 0.5 mg/ml) in 0.05*M* tris-HCl, *p*H 8.1, containing 0.02*M* $CaCl_2$, was incubated with soybean inhibitor (16 and 8 μg, respectively) for 5 minutes. TAME (0.3 ml of a 0.01*M* solution in water) was then added to bring the final volume to 3.0 ml. The extinctions were measured at 247 nm against a blank containing protein in one side and TAME in the other side of a divided cuvette. Solid circles and squares, acetyltrypsinogen treated with DFP; open circles and squares, not treated with DFP. At the higher concentrations, the curves are asymptotic as the hydrolysis approached completion (absorbance approximately 0.55). [From Kay and Kassell (*27*)] [Permission of the American Society of Biological Chemists]

extended the lag phase observed before detectable tryptic activity appeared, but did not prevent the full activation of the trypsinogen. The results with soybean inhibitor, presented in Table 1, show both the effect of trypsinogen concentration on the initiation of autoactivation and the delaying effect of the inhibitor. These results suggested that trypsinogen catalyzed its own activation in a concentration-dependent reaction, but the presence of a few molecules of contaminating trypsin could not be absolutely excluded.

In the second set of experiments we utilized a system in which there would be no possibility of the reaction product accelerating the reaction, and in which a trace of trypsin would not invalidate the results. Acetyltrypsinogen cannot be activated by trypsin (*30*) because the ξ-amino group of the lysine residue at the bond normally split during activation is acetylated and is no longer susceptible to tryptic digestion. Acetyltrypsin, however, is fully active (*31*). Acetyltrypsinogen was prepared and a portion was kept for several days under activating conditions. No activity could be detected, and no increase in activity was obtained on addition of exogenous trypsin. Therefore, the question that remained was whether or not this trypsinogen derivative that could not be activated to trypsin had inherent activity.

Two methods were used to test for activity of the acetyltrypsinogen: activation of chymotrypsinogen (a normal function of trypsin) and hydrolysis of a synthetic trypsin substrate. The acetyltrypsinogen was able to activate chymotrypsinogen A (open squares in Fig. 6) and to hydrolyze tosylarginine methyl ester, the trypsin substrate (open circles and open squares in Fig. 7). The rates were slow in both reactions; chymotrypsinogen activation proceeded at about 1 percent per day, and the hydrolysis of tosylarginine methyl ester at approximately 10^{-5} the rate with trypsin.

If the trypsinogen preparation contained contaminating trypsin, this would have been converted to acetyltrypsin during the acetylation step. Treatment of the acetyltrypsinogen with DFP should then remove most, if not all, of the acetyltrypsin, and a significant difference should be seen in the *rates* of reaction between DFP-treated and untreated samples. Figures 6 and 7 show that there was no significant difference in the experiments with and without DFP. Thus the activity of acetyltrypsinogen was not due to contamination by free enzyme, but was due to an inherent activity of the zymogen.

We can therefore conclude that trypsinogen does have an inherent capacity to activate itself, albeit at a very much slower rate than the activation catalyzed by trypsin or enterokinase. This self-activation is probably an intermolecular reaction, because the lag time increased on decreasing the concentration of trypsinogen (Table 1). This idea is given further support by the activation of chymotrypsinogen and the hydrolysis of tosylarginine methyl ester by acetyltrypsinogen. These must be intermolecular reactions.

The formation of "active" trypsinogen molecules can be envisaged in two ways. First, a trypsinogen molecule may undergo a structural alteration that produces an active species that can then act on other "substrate" molecules of trypsinogen. This is not improbable, since it has already been shown (*32*) by physical methods that trypsinogen can undergo several reversible transitions that have the effect of producing a structure more like that of trypsin. Second, a bimolecular interaction may occur [analogous to that proposed for ribonuclease (*33*)] in which two trypsinogen molecules come together momentarily, perhaps with conformational alterations, with one contributing the

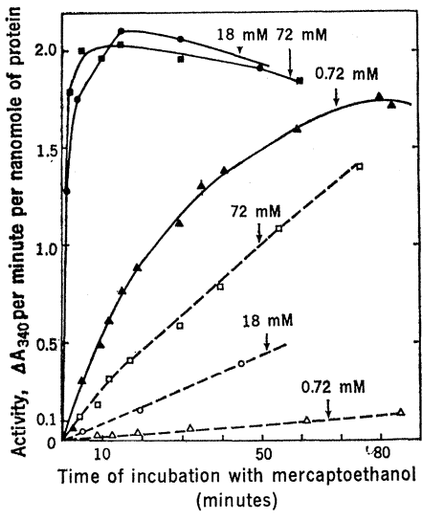

Fig. 8. Activation of the zymogen (open symbols) and proteolyzed zymogen (solid symbols) of streptococcal proteinase by various concentrations of mercaptoethanol at *p*H 7. Protein concentration was about 50 μg/ml. Esterase activity was determined with *N*-α-benzyloxycarbonyl-L-lysine hydrochloride *p*-nitrophenyl ester as substrate. [From Bustin *et al.* (*11*)] [Permission of the American Society of Biological Chemists]

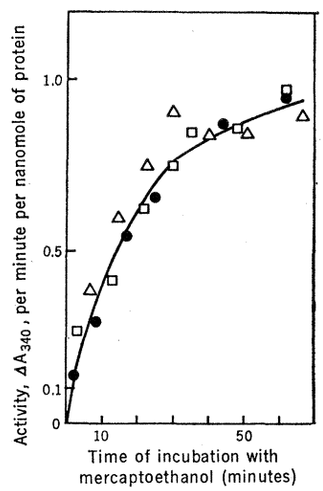

Fig. 9. Lack of effect of protein concentration on the course of activation of zymogen. After 15 minutes of incubation at 25°C and *p*H 7, the incubation mixture was made 72.5 m*M* in mercaptoethanol. Zymogen concentrations: open triangles, 11 μg per milliliter; open squares, 27 μg/ml; solid circles, 55 μg/ml. Activity was determined as in Fig. 8. [From Bustin *et al.* (*11*)] [Permission of the American Society of Biological Chemists]

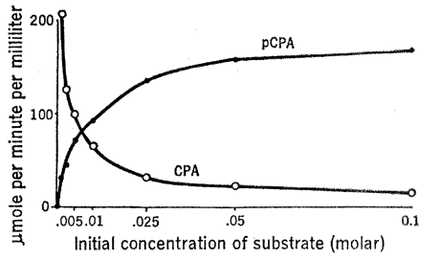

Fig. 10. Substrate concentration dependence of the hydrolysis of hippuryl-DL-β-phenyllactic acid by procarboxypeptidase A (*pCPA*) and carboxypeptidase A (*CPA*). [From Lacko and Neurath (*41*)] [Permission of the American Chemical Society]

imidazole group of the active histidine and the other the hydroxyl group of the active serine. This dimerization may cause the formation, in an occasional molecule, of a trypsin-like active site that can then split off the activation peptide from one or the other of these molecules or from a third trypsinogen molecule to form active trypsin. Dimerization is a reasonable assumption, since chymotrypsin forms dimers and higher aggregates (*34*).

In these experiments with trypsinogen we were concerned with demonstrating activity in this zymogen. More recently, two quite different methods have confirmed the existence of an active site in the zymogens of "serine" proteases.

By treating trypsinogen for several hours with a high concentration of radioactive DFP, Morgan *et al.* (*35*) were able to incorporate the diisopropyl phosphoryl group almost specifically into the serine of the active site. The rate of reaction was much less than with trypsin (as we had found with other reactions), the second order rate constants being 0.041 and 300 liter mole^{-1} min^{-1} for trypsinogen and trypsin, respectively. The alkylated zymogen could not be activated. Chymotrypsinogen also reacted with DFP, again at a much slower rate than chymotrypsin. Thus, chemical modification experiments with these zymogens provide evidence that they contain an inherent active site.

Robillard and Shulman (*36*) used nuclear magnetic resonance in their studies of chymotrypsinogen. They found a single resonance at very low magnetic fields, which they assigned to the hydrogen-bonded proton between histidine-57 and aspartic acid-102. Both of these amino acids are part of the charge-relay system believed to participate along with serine in the catalytic activity of chymotrypsin (*37*). The important point is that this resonance was present in both chymotrypsin and chymotrypsinogen, and is thus another proof of a preformed active site in the zymogen.

A Sulfhydryl Protease

Proteolytic enzymes that have a sulfhydryl group at the active site are common in nature, for example, the plant protease, papain, some of the cathepsins of mammalian tissues, and numerous bacterial proteases. These enzymes are reversibly inactivated by air oxidation of the sulfhydryl group to the disulfide state, and are reactivated by reducing agents.

The first instance of the occurrence of a zymogen of a bacterial sulfhydryl protease was reported by Elliott (*10*). An extracellular protease of group A streptococci is formed from a zymogen containing a single disulfide bond. This is an unusual mixed disulfide derived from a half-cystine residue and an unknown volatile mercaptan (*38*). Like the mammalian enzyme precursors, this zymogen undergoes autoactivation in which a portion of the protein is removed by proteolysis. However, in this case, activity appears only in the presence of a reducing agent and the active enzyme has a free sulfhydryl group (*39*). Thus, there are two steps in the activation, proteolysis and reduction, and the question of whether the reduced unproteolyzed zymogen had any proteolytic activity arose (*40*).

Autodigestion, with release of peptides from the zymogen, occurs rapidly in concentrated solutions (approximately 2 mg per milliliter). Controlled hydrolysis with trypsin produces a proteolyzed zymogen that becomes an active enzyme when its disulfide bond is reduced (*39*), so that a convenient inactive form of the enzyme is available for testing. Bustin *et al.* (*11*) compared the reduction of native zymogen with this proteolyzed zymogen in dilute solutions (20 to 50 µg/ml). Figure 8 illustrates these experiments and shows the rate of appearance of activity with different concentrations of reducing agent. The intact zymogen was less susceptible to reduction, but at the highest concentration of mercaptoethanol, after reduction for 75 minutes, the native zymogen approached the full activity of the proteolyzed reduced zymogen.

There is evidence that proteolysis did not occur in such dilute solution during reduction. The rate of appearance of activity was dependent only on the concentration of mercaptoethanol (Fig. 8), not on the concentration of protein (Fig. 9). Therefore, a bimolecular reaction between a trace of reduced proteolyzed zymogen and zymogen or between molecules of the reduced zymogen was not the cause of the activation. The elution profile following gel filtration of the reduced zymogen gave no indication of peptides such as those formed by autoactivation in concentrated solutions of zymogen. Most significant is the fact that the amino acid composition of the reduced active zymogen was not different from the starting material; no peptides had been split off.

These experiments, together with several others (*11*), provide strong evidence that this is another zymogen that may be active without the removal of activation peptides.

A Metalloenzyme

Carboxypeptidases are enzymes that hydrolyze amino acids one at a time from the carboxyl terminal of proteins and peptides. They are metalloenzymes and normally contain zinc at their active sites.

Procarboxypeptidase A from dogfish pancreas is converted to carboxypeptidase A by trypsin. Lacko and Neurath (*41*) made the interesting observation that the dogfish zymogen is 2 to 25 percent as active as the enzyme when acting on peptide or ester sub-

Table 2. Comparison of the kinetic constants (K_m, Michaelis constant; K_{cat}, catalytic constant) of dogfish procarboxypeptidase A and carboxypeptidase A. [From Lacko and Neurath (*41*)] [Permission of the American Chemical Society]

Enzyme	Hippuryl-DL-β-phenyllactic acid		Benzyloxycarbonyl-glycyl-L-phenylalanine		*N*-acetylglycyl-L-phenyllactic acid	
	K_m (m*M*)	K_{cat} (sec^{-1})	K_m (m*M*)	K_{cat} (sec^{-1})	K_m (m*M*)	K_{cat} (sec^{-1})
Carboxypeptidase	0.02	635	0.21	14.7	0.44	86
Procarboxypeptidase	8	106	1.8	1.2	12	24

strates (Table 2), suggesting that a true catalytic site is operational in the zymogen. Increasing concentrations of hippuryl-β-phenyllactic acid showed strong substrate inhibition with the enzyme, but not with the zymogen (Fig. 10). Thus, at high substrate concentrations, the zymogen is more active than the enzyme! With another substrate, benzyloxycarbonylglycylphenylalanine, the enzyme showed substrate activation, while the zymogen again showed no effect. The conclusion from these experiments is that the enzyme has a more efficient binding site for substrates than the zymogen, rather than a more effective catalytic site.

When cobalt was substituted for zinc in bovine procarboxypeptidase A (*42*), this zymogen was also more active than the corresponding cobalt enzyme on several substrates.

Conclusions

The idea that the precursors of proteolytic enzymes have inherent activity first appeared in print in 1966 (*12*). Nevertheless, investigations of the zymogens of all four types of proteolytic enzymes were already, entirely independently, planned or in progress in several laboratories. All investigators devised special techniques to ensure that it was the zymogen and not the enzyme that was responsible for the activity. Evidence of inherent activity was found for all the different kinds of zymogens, but the rate of action, in comparison to the enzyme, varied from a barely detectable amount in the case of trypsinogen to activity even greater than the enzyme under certain conditions for procarboxypeptidase A.

For zymogens that undergo autoactivation to form their enzymes, such as pepsinogen, prorennin, and trypsinogen, it is now clear that they can initiate their own activation and are not dependent on a trace of active enzyme as was formerly thought to be the case. Autoactivation, previously considered to be a reaction in which the enzyme produced more of itself, should now be redefined as a process initiated by the enzyme precursor and then accelerated by the active enzyme formed. The sequence of events is probably a conformational change resulting in active zymogen, followed by the proteolytic action of this active zymogen on other zymogen molecules, converting them to enzyme.

The data discussed herein suggest some interesting prospects for further work. A highly active zymogen such as dogfish procarboxypeptidase A, unlike the enzyme in not being subject to substrate inhibition, may be useful for sequence work on proteins. Kinetic comparisons of other zymogens with their enzymes, following the studies on procarboxypeptidase A, should provide information about the relationships of the binding sites to the catalytic sites. For example, do the zymogens have a broader specificity than their enzymes? Other systems, not yet investigated from this point of view, may also turn out to be self-starting, for example, the blood-clotting cascade. Perhaps the zymogens of some nonproteolytic enzymes, such as prophospholipase, also have inherent activity on appropriate substrates.

References and Notes

1. R. Heidenhain, in *Handbuch der Physiologie*, vol. 5, L. Hermann, Ed. (Vogel, Leipzig, 1883), p. 173; G. E. Palade, P. Siekevitz, L. G. Caro, in *Ciba Foundation Symposium on the Exocrine Pancreas*, A. V. S. de Reuck and M. P. Cameron, Eds. (Churchill, London, 1962), p. 23; L. J. Greene, C. H. W. Hirs, G. E. Palade, *J. Biol. Chem.* **238**, 2054 (1963).
2. R. M. Herriott, *J. Gen. Physiol.* **22**, 65 (1938).
3. M. Kunitz and J. H. Northrop, *ibid.* **19**, 991 (1936); J. H. Northrop, M. Kunitz, R. M. Herriott, *Crystalline Enzymes* (Columbia Univ. Press, New York, 1948.)
4. S. Maroux, J. Baratti, P. Desnuelle, *J. Biol. Chem.* **246**, 5031 (1971).
5. M. Ottesen, *Annu. Rev. Biochem.* **36**, 55 (1967); R. Kleine, *Naturwiss. Rundsch.* **23**, 94 (1970).
6. D. F. Steiner, D. Cunningham, L. Spigelman, B. Aten, *Science* **157**, 697 (1967); R. E. Chance and R. M. Ellis, *Arch. Intern. Med.* **123**, 229 (1969).
7. E. W. Davie, C. Hougie, R. L. Lundblad, in *Recent Advances in Blood Coagulation*, L. Poller, Ed. (Churchill, London, 1969), p. 13.
8. G. H. De Haas, F. Franček, B. Keil, D. W. Thomas, E. Lederer, *Fed. Eur. Biochem. Soc. Lett.* **4**, 25 (1969); J.-P. Abita, M. Lazdunski, P. P. M. Bonsen, W. A. Pietersen, G. H. De Haas, *Eur. J. Biochem.* **30**, 37 (1972).
9. R. S. Yalow and S. A. Berson, *Gastroenterology* **58**, 609 (1970).
10. S. D. Elliott, *J. Exp. Med.* **92**, 201 (1950).
11. M. Bustin, M. C. Lin, W. H. Stein, S. Moore, *J. Biol. Chem.* **245**, 846 (1970).
12. B. Foltmann, *Compt. Rend. Trav. Lab. Carlsberg* **35**, 143 (1966).
13. ———, in *Milk Proteins*, H. A. McKenzie, Ed. (Academic Press, New York, 1971), p. 217.
14. R. C. Wong, Y. Nakagawa, G. E. Perlmann, *J. Biol. Chem.* **247**, 1625 (1972).
15. T. D. Tanksley, Jr., H. Neumann, C. M. Lyman, C. N. Pace, J. M. Prescott, *ibid.* **245**, 6456 (1970).
16. H. Van Vunakis and R. M. Herriott, *Biochim. Biophys. Acta* **23**, 600 (1957); E. B. Ong and G. E. Perlmann, *J. Biol. Chem.* **243**, 6104 (1968).
17. M. Bustin and A. Conway-Jacobs, *J. Biol. Chem.* **246**, 615 (1971).
18. J. Al-Janabi, J. A. Hartsuck, J. Tang, *ibid.* **247**, 4628 (1972).
19. M. Funatsu, Y. Harada, K. Hayashi, B. Jirgensons, *Agr. Biol. Chem.* **35**, 566 (1971).
20. M. Funatsu, Y. Harada, K. Hayashi, T. Kaneda, *ibid.* **36**, 305 (1972).
21. P. McPhie, *J. Biol. Chem.* **247**, 4277 (1972).
22. J. L. Wang and G. M. Edelman, *J. Biol. Chem.* **246**, 1185 (1971).
23. Abbreviations: Val, valine; Asp, aspartic acid; Lys, lysine.
24. E. W. Davie and H. Neurath, *J. Biol. Chem.* **212**, 515 (1955).
25. H. Neurath and G. H. Dixon, *Fed. Proc.* **16**, 791 (19[illegible] P. B. Sigler, D. M. Blow, B. W. Matthews, R. Henderson, *J. Mol. Biol.* **35**, 143 (1968).
26. J. Kay and B. Kassell, *Fed. Proc.* **30**, 1077 (1971).
27. ———, *J. Biol. Chem.* **246**, 6661 (1971).
28. J. Lebowitz and M. Laskowski, Jr., *Biochemistry* **1**, 1044 (1962).
29. G. H. Dixon, D. L. Kauffman, H. Neurath, *J. Amer. Chem. Soc.* **80**, 1260 (1958).
30. J.-F. Pechère and H. Neurath, *J. Biol. Chem.* **229**, 389 (1957).
31. L. Terminiello, J. Sri Ram, M. Bier, F. F. Nord, *Arch. Biochem. Biophys.* **57**, 252 (1955).
32. M. Lazdunski and M. Delaage, *Biochim. Biophys. Acta* **140**, 417 (1967); M. Delaage and M. Lazdunski, *Biochem. Biophys. Res. Commun.* **28**, 390 (1967); R. Chicheportiche and M. Lazdunski, *Fed. Eur. Biochem. Soc. Lett.* **3**, 195 (1969).
33. A. M. Crestfield, W. H. Stein, S. Moore, *J. Biol. Chem.* **238**, 2421 (1963).
34. The original observation was made by G. W. Schwert, *J. Biol. Chem.* **179**, 655 (1949); extensive work since that time is summarized by K. C. Aune and S. N. Timasheff, *Biochemistry* **10**, 1609 (1971).
35. P. H. Morgan, N. C. Robinson, K. A. Walsh, H. Neurath, *Proc. Nat. Acad. Sci. U.S.A.* **69**, 3312 (1972).
36. G. Robillard and R. G. Shulman, *J. Mol. Biol.* **71**, 507 (1972).
37. D. M. Blow, J. J. Birktoft, B. S. Hartley, *Nature* **221**, 337 (1969).
38. T.-Y. Liu, N. P. Neumann, S. D. Elliott, S. Moore, W. H. Stein, *J. Biol. Chem.* **238**, 251 (1963); W. Ferdinand, W. H. Stein, S. Moore, *ibid.* **240**, 1150 (1965).
39. T.-Y. Liu and S. D. Elliott, *ibid.* **240**, 1138 (1965).
40. ———, *Nature* **206**, 33 (1965).
41. A. G. Lacko and H. Neurath, *Biochemistry* **9**, 4680 (1970).
42. W. D. Behnke and B. L. Vallee, *Fed. Proc.* **31** (2), 435 (1972).

43. The research from this laboratory was supported by the National Science Foundation (GB-12630) and by the U.S. Public Health Service (grant AM-09826 from the National Institute of Arthritis and Metabolic Diseases).