Protein Degradation at Neutral pH. Possible Enzymic and Control Mechanisms[1] (35826)

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Several investigators (1–3) have shown that in tissue slices the process of protein degradation at neutral pH can be significantly reduced by the uncoupling or inhibition of the oxidative phosphorylation. Complementary studies have indicated that the addition of adenosine-5′-triphosphate (ATP) to preparations of intracellular organelles (4) or to whole tissue homogenates (5, 6) greatly increase the rate of proteolysis, and that this activating effect is mediated by aspartic acid, presumably through the formation of an adenylate derivative (7). This activating effect of ATP was found to be widespread in the organs of the rat (6) and to be able to return to a normal level the reduced proteolytic activity of the liver of rats subjected to an acute stress (8).

All these studies indicate that not only is there a regulatory mechanism for the control of protein degradation at neutral pH, but also that the same mechanism may be involved in the correlation of the rate of protein degradation with the rate of protein synthesis during turnover. This possibility has increased the need to elucidate the nature of the proteolytic enzymes responsible for the neutral proteolytic activity and particularly, the need to identify and characterize the enzyme or enzymes activated by ATP.

This article describes an effort to determine the main types of proteolytic enzymes active in liver homogenates at pH 7.5 and describes their behavior toward ATP during 2 hr of autolysis. These studies have allowed the formulation of an hypothesis, which provides a mechanism for the control of neutral proteolysis and for the interaction of this process with the process of protein synthesis.

Materials and Methods. These studies were carried out with 10% liver homogenates made in 0.22 *M* phosphate buffer (pH 7.5)–0.2% Triton X-100. The amount of protein in the homogenate was determined according to the method of Lowry *et al.* (9) using bovine serum albumin as standard.

Studies with synthetic substrates and modified albumin. Qualitative studies were carried out with 50 m*M* solutions of the following synthetic substrates[3]: L-leucine amide, L-phenylalanine amide, glycyl-L-phenylalanine, glycyl-L-phenylalanine amide, glycyl-L-phenylalanyl-L-phenylalanine, L-phenylalanyl-L-phenylalanine, L-phenylalanyl-L-tyrosine, L-leucyl-L-tyrosine, L-tyrosyl-L-leucine, *N*-carbobenzoxy-glycyl-L-tyrosine, *N*-carbobenzoxy-L-glutamyl-*L*-tyrosine, and α-*N*-benzoyl-L-arginine amide. Two hundred microliter of a peptide solution were incubated for 2 hr at 37° with the same volume of a 4% homogenate made by diluting a 10% homogenate with 0.22 *M* phosphate buffer, pH 7.5. At this time 200 μl of 10% tricholoroacetic acid (TCA) were added, and 50 μl of the supernatant were spotted on a 20 × 10-in. sheet of Whatman No. 1 paper. For identification purposes a similar volume of a 5 m*M* reference solution of each one of the possible products of hydrolysis of the peptide in question was simultaneously spotted. The constituents in the spots were separated with a solvent mixture containing *n*-butanol:acetic

[1] This work was accomplished with the support of the School of Dentistry of the University of Minnesota.

[2] The author gratefully acknowledges the technical assistance of Mr. Daniel Mills.

[3] These compounds were obtained from Sigma Chemical Company, St. Louis, Missouri.

acid:pyridine:water (15:3:10:12) by descending chromatography after 6 hr of equilibration. The formation of Ninhydrin-positive products other than the products of hydrolysis of the synthetic peptides was monitored by spotting the TCA supernatant of incubation mixtures in which the liver homogenate had been previously heated at 95° or in which the substrate solution had been substituted for phosphate buffer. The products were developed with a Ninhydrin spray and were identified by comparing their rate of migration with the rate of the appropriate reference solutions.

The quantitative determination of aminopeptidase activity was carried out with L-leucine amide and L-phenylalanine amide as substrates by the microdiffusion technique of Conway (10). The assay for carboxypeptidase activity was carried out with *N*-CBZ-glycyl-L-tyrosine as substrate according to the method described by Iodice *et al.* (11).

The preparation of the albumin substrate for the assay of the overall proteolytic activity has been already described (7). The acetylation of this substrate was carried out according to the method described by Riordan and Vallee (12). The degree of acetylation was determined by comparing the amount of Ninhydrin color obtained by the method of Spies (13) from the albumin substrate before and after acetylation. The degree of acetylation obtained varied between 90 to 95%. After completion of the acetylation, the substrate was extensively dialyzed against 0.22 *M* phosphate buffer (pH 7.5) and then made 0.005 *M* in iodoacetamide[3] and 0.1 *M* in EDTA.[3]

The assay of the proteolytic activity with this substrate was carried out in incubation mixtures containing 3 ml of substrate solution and 3 ml of 10% homogenate. Two-milliliter samples were taken before and after 30 min of incubation at 37° and pipetted into 1 ml of 10% TCA. The amount of Ninhydrin-positive material released into the TCA supernatant was determined by the method of Spies (13) using L-tyrosine as standard. The amount of proteolysis was calculated by subtracting the amount of Ninhydrin color in the first aliquot from that in the 30-min aliquot. These conditions provided a direct relationship between the amount of liver protein in the mixture and the amount of product formed. The results were expressed as micromoles of L-tyrosine released per gram of liver protein under the stated conditions.

Studies using acetylated albumin and the B-chain of insulin as substrate. The B-chain of insulin was prepared by oxidation of crystalline bovine insulin[3] and isolated according to method described by Sanger (14). Electrophoretic studies of the final product showed only a negligible amount of contaminant in the form of a faster moving component.

In one type of experiment, a 10% liver homogenate was made 0.1 *M* in EDTA, 0.005 *M* in iodoacetamide, and 1 *M* in butanol. After 30-min standing, the precipitated protein was centrifuged out and 0.5 ml of the clear solution (0.11 mg of total protein) were incubated with 5 ml of acetylated albumin for 3.5 hr at 37°. At this time 3 ml of 10% TCA were added to stop the reaction. The precipitated protein was centrifuged down and after filtering the clear supernatant through Whatman No. 1 paper, the water and TCA were completely evaporated in vacuum. The residue was redissolved in 0.001 *M* phosphate buffer (pH 7.5) and was fractionated in a Sephadex CM column using a 0.001–0.22 *M* linear gradient of phosphate buffer (pH 7.5) for elution. As a control, an identical study was carried out using nonacetylated albumin as substrate.

In complementary studies, two flasks containing 80 mg of the B-chain of insulin (4 ml) and 8 mg of liver protein (0.5 ml of liver homogenate) were incubated for 2 and 8 hr respectively. At this time the undegraded proteins were precipitated by the addition of TCA. The clear supernatants were dried in vacuum and resuspended in 0.5 ml of 0.2 *M* citrate buffer, pH 2.2. The free amino acids in this solution were quantitatively determined in a Beckman C-120 Automatic Amino Acid Analyzer.[4] The results were expressed

[4] The amino acid analyses were carried out in the laboratories of Dr. Ulisses Seal of the Veterans Administration Hospital of Minneapolis, for which the author expresses his gratitude.

TABLE I. Degradation of Synthetic Peptides by Rat Liver Homogenates Adjusted to pH 7.5.

Substrate	EDTA, 0.1 *M*	Mg^{2+}, 1 m*M*	Iodoacetamide, 0.005 *M*	Butanol, 1 *M*
Amino peptidases				
1. Leu NH_2 → Leu + NH_4^+	Inh	Act	—	—
2. Phe NH_2 → Phe + NH_4^+	Inh	Act	Inh	—
3. Gly Phe → Gly + Phe	Inh	—	—	—
4.[a] Gly Phe NH_2 → Gly + Phe NH_2	—	—	—	Inh
5. Gly Phe Phe → Gly + Phe Phe	—	—	—	Inh
6. Phe Phe → 2 Phe	—	—	—	Inh
7. Phe Tyr → Phe + Tyr	—	—	—	Inh
8. Leu Tyr → Leu + Tyr	—	—	—	Inh
9. Tyr Leu → NR				
Carboxypeptidases				
10. CBZ–Gly–Tyr → GBZ Gly + Tyr	—	—	Inh	—
Known intracellular proteases				
11. Gly–Phe–NH_2 → Gly Phe + NH_4^+ (cathepsin C)	—	—	Inh	—
12. CBZ–Glut–Tyr → NR (cathepsin A)				
13. BZ–Arg–NH_2 → NR (cathepsin B)				

[a] Nos. 4–9 Studied after 48 hr of dialysis against EDTA and in the presence of iodoacetamide.

as the number of amino acid residues released with respect to the one in the lowest concentration, L-tyrosine.

Studies with liver autolysates. The process of autolysis in liver homogenates was followed by determining the amount of TCA-soluble Lowry-positive and Ninhydrin-positive material liberated during 2 hr of incubation. For this purpose, 20 ml of a 10% homogenate were incubated at 37° in a constant temperature bath. After 5 min of incubation and every 20 min thereafter, 2-ml samples were taken and pipetted into 1 ml of 10% TCA. The amount of products released was determined with Ninhydrin and the Lowry reagent as previously described (7). To study the changes in the activity of specific proteases during autolysis, the 2-ml samples were kept in ice instead of being precipitated with TCA. After completion of the 2 hr of incubation, the activities were assayed as described above. The change in activity was expressed as percentage change with respect to the activity at zero time.

In some experiments, 1 ml of a preparation of "amino acid activating enzymes" (1.1 mg of total protein) prepared according to the method of Hoagland *et al.* (15) was added to 20 ml of liver homogenate after 80 min of incubation. In some cases, 1 mg of ATP was simultaneously added with the "activating enzymes" and at 20-min interval for the rest of the experiment. The intrinsic proteolytic activity of the "activating enzymes" preparation has been found to be negligible (7). The results in all the autolysis experiments have been expressed as either milligrams of albumin equivalents or micromoles of tyrosine liberated per gram of liver protein, depending upon the colorimetric procedure used.

Results and Discussion. The hydrolysis of synthetic peptides by the liver homogenates (Table I) showed the presence of several types of aminopeptidase activity, among them a magnesium dependent leucine aminopeptidase, a magnesium dependent iodoacetamide inhibited aminopeptidase, a metalloenzyme not activated by magnesium, and an aminopeptidase active in the presence of either EDTA or iodoacetamide but inhibited

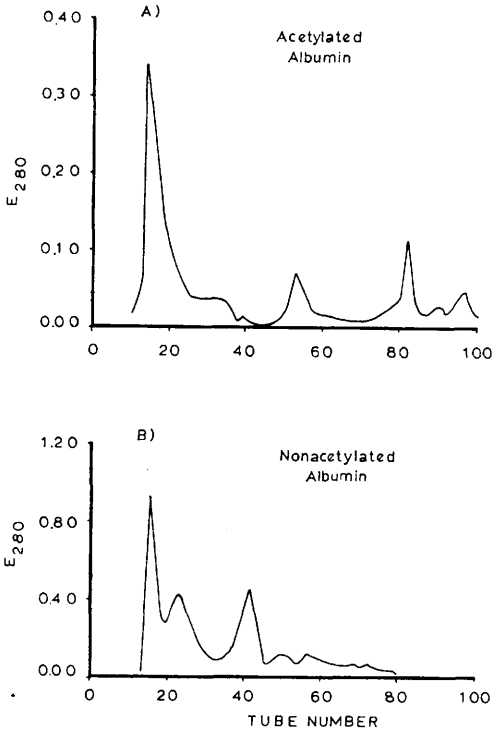

FIG. 1A. Fractionation of the TCA-soluble material released from acetylated albumin by a 10% pH 7.5 liver homogenate containing 0.005 *M* iodoacetamide, 0.1 *M* EDTA, and 1 *M* butanol. (B) Fractionation of the TCA-soluble material released from nonacetylated albumin by the same homogenate used in (A). Both fractionations were carried out at room temperature in a Sephadex-CM column, 40 × 3.5 cm, eluted with a linear gradient 0.001–0.22 *M* of phosphate buffer (pH 7.5) at a rate of 30 ml/hr. All the peaks were ninhydrin- and Lowry-positive and migrated on paper chromatography at a different rate than the UV absorbing amino acids. All of them produced, upon acid hydrolysis and subsequent chromatography on paper, several Ninhydrin-positive spots.

by 1 *M* butanol.

The hydrolysis of *N*-CBZ-glycyl-L-tyrosine showed the presence of a carboxypeptidase system inhibited by iodoacetamide.

Of the substrates commonly used to detect the activity of cathepsin A (CBZ–Glut–Tyr), B or B^1 (BZ–Arg–NH_2) and C (Gly–Phe–NH_2), only the substrate for cathepsin C was split under our experimental conditions. No cathepsin B activity could be detected after addition of cysteine. The lack of a specific synthetic substrate for cathepsin D prevented the direct investigation of the activity of this enzyme. However, since the studies of Press *et al.* (16) clearly showed that this cathepsin is inactive above pH 5.5, it seems unlikely that it would play any role in neutral proteolysis.

After complete inhibition of all the activities presented in Table I by intensive dialysis against 0.22 *M* phosphate buffer (pH 7.5–0.1 *M* EDTA, and by subsequent addition of iodoacetamide up to a 0.005 *M* concentration and *n*-butanol to a concentration of 1 *M*, the clear solution obtained after removal of the precipitated protein was found still capable of degrading acetylated albumin. Since the *N*-terminal positions of this substrate had been already blocked by the addition of the acetyl group, and since the carboxypeptidase activity had been completely inhibited by the addition of iodoacetamide, it was necessary to conclude that the formation of the TCA soluble products represented in Fig. 1A was probably due to the presence of neutral endopeptidases. The peptide nature of the products (Fig. 1A) was confirmed by their positive reaction with the Ninhydrin and Lowry reagents, by their different rate of migration in paper chromatography from that of the UV absorbing amino acids (280 mμ), and by the formation of new and multiple Ninhydrin-positive spots detectable by paper chromatography after complete hydrolysis with HCl.

The different elution pattern obtained when the TCA-soluble material released from the nonacetylated albumin (Fig. 1B) was chromatographed in Sephadex-CM, and particularly the absence of the small molecular weight peaks which because of their size are more susceptible to the attack of aminopeptidases, suggests that the treated homogenates still contained some aminopeptidases not susceptible to the effect of the added inhibitors. Under these conditions, the peptides isolated in Fig. 1A are the result, not only of the attack of the acetylated albumin by the endopeptidases, but also of a secondary degradation of these original peptides by the unidentified aminopeptidases.

The amino acids released by the action of the liver proteases from the B-chain of insulin are presented in Table II. The first

TABLE II. Amino Acid Residues Released at Neutral pH from the B-Chain of Insulin by Liver Homogenates After 2 and 8 hr of incubation at 37°.

Residue	No. of residues released	
	2 hr	8 hr
Phenylalanine	7	18
Valine	3	14
Leucine	2	8
Asparagine	4	6
Alanine	1	5
Tyrosine	—	5
Gylcine	—	2
Glutamic acid	—	3
Histidine	—	3
Lysine	—	1
Threonine	—	1
½ Cystine	—	2
Serine	—	1

column gives the number of residues released after 2 hr and the second one gives the number of residues released after 8 hr of incubation.

Figure 2 represents the primary structure of the B-chain showing the peptide bonds that had to be hydrolyzed to obtain the amino acids presented in Table II. The distribution of the arrows indicates that the B-chain was degraded by the concerted action of endo and exopeptidases, the endopeptidase attack occurring somewhere between residues 9 and 19, most probably between 13 and 16 according to the 2-hr experiment, and between residues 23 and 26.

The behavior during 2 hr of autolysis of two aminopeptidase systems, the carboxypeptidase activity and the endopeptidase activity as measured by the degradation of acetylated albumin in the presence of EDTA and iodoacetamide are presented in Fig. 3. As shown, the aminopeptidase activities remained constant during most of the autolysis period while at the end of this period the endopeptidase and the carboxypeptidase activities had decreased to 50% of the initial value in the case of the endopeptidase and to undetectable levels in the case of the carboxypeptidase.

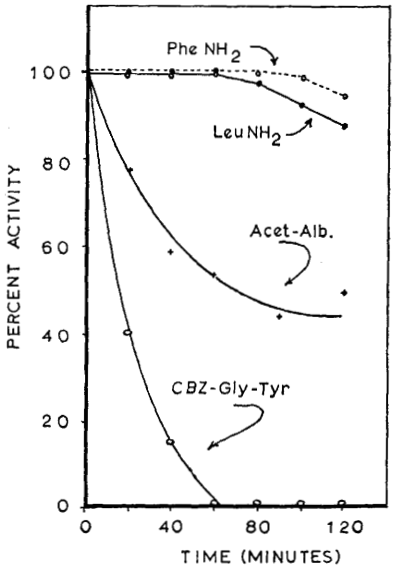

FIG. 3. Changes in two aminopeptidase activities (Phe NH_2 and Leu NH_2 used as substrates), the endopeptidase activity (acetylated albumin used as substrate) and the carboxypeptidase activity (CBZ–Gly–Tyr used as substrate) during 2 hr of autolysis at pH 7.5: Each point corresponds to the average of two different experiments.

The pattern of release of the TCA-soluble autolysis products as detected by the Ninhy-

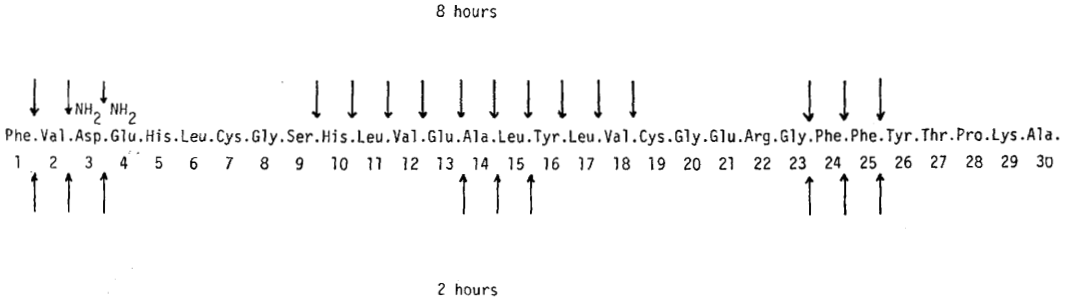

FIG. 2. Primary structure of the B-chain of insulin showing the bonds that had to be hydrolyzed by the neutral proteases to obtain the amino acid residues presented in Table II.

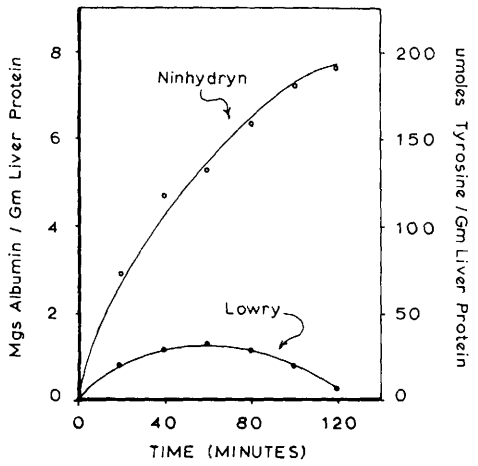

FIG. 4. Release of TCA-soluble Lowry-positive and Ninhydrin-positive material during 2 hr of autolysis of a 10% (pH 7.5) liver homogenate incubated at 37°: Each point corresponds to the average of five different experiments.

drin and Lowry reagents is presented in Fig. 4. In Fig. 4, it is interesting to note that, even though the Ninhydrin color increased constantly throughout the incubation period, the release of Lowry-positive material leveled off after 40 min of incubation and finally declined to values close to zero at the end of the incubation period. These changes in the Lowry color can be associated with the slow decline in the endopeptidase activity (Fig. 3) in the presence of an almost constant aminopeptidase activity and suggest that during autolysis, the endopeptidase activity becomes progressively inhibited, probably by its own products, until the rate of formation of peptides and the rate of their subsequent degradation to amino acids by the exopeptidases reaches a steady state. This point corresponds to the plateau in the Lowry curve and to a constantly increasing Ninhydrin curve. A further decline in the amount of active endopeptidase with a still high aminopeptidase activity (Fig. 3) eventually upsets the steady state, forcing the degradation of the peptides accumulated during the initial stage of autolysis and accounting not only for the disappearance of the Lowry color but also for the continuous increase in the Ninhydrin color.

The addition of ATP to the homogenates during autolysis has been shown to increase the rate of protein degradation (7). When the behavior of the proteases presented in Fig. 3 was studied in the presence and in the absence of ATP, only the endopeptidase activity was found to be increased (Fig. 5).

In agreement with the previous experiment, the steady state between endopeptidases and the aminopeptidases represented by the plateau in the Lowry color was found to be upset in favor of the endopeptidases, indicated by the increase in the Lowry color, by either the addition of "amino acid activating enzymes" alone, or by the simultaneous addition of these enzymes and ATP (Fig. 6). Since "free" amino acids are abundantly present in this partially autolyzed homogenate, these results lend further support to our previous work (7) in which an aminoacyl adenylate has been suspected as the enzyme activator.

The experiments conducted with the B-chain of insulin (Fig. 2) suggested that proteins are degraded by the neutral proteases, not only by the attack of aminopeptidases as proposed by Marrink and Gruber (17), but

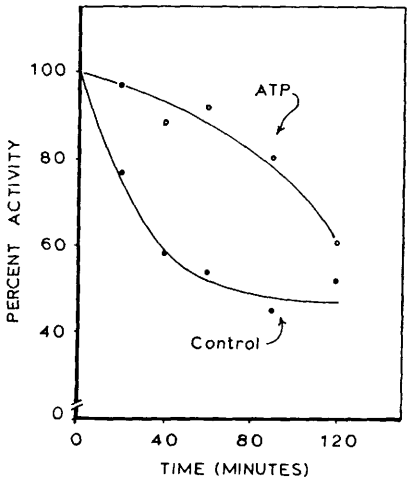

FIG. 5. Change in endopeptidase activity (acetylated albumin used as substrate) in the presence and absence of exogenous ATP, during 2 hr of autolysis of a 10% (pH 7.5) liver homogenate: The ATP was added to the autolyzing homogenate every 20 min during the experimental period. Each point corresponds to the average of 3 different experiments.

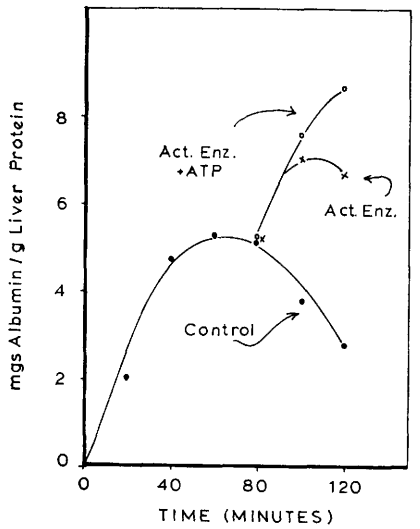

FIG. 6. Reactivation of the production of Lowry-positive material after 80 min of autolysis of a neutral homogenate by the addition of a small amount of "amino acid activating enzymes" with, or without exogenous ATP: Each point corresponds to the average of two different experiments.

also by the hydrolysis of internal bonds and the subsequent degradation of the newly formed peptides by the action of both amino-peptidases and carboxypeptidases.

As a summary, Fig. 7 presents a probable enzymic mechanism for the neutral degradation of protein and its possible regulation by an ATP derivative. This scheme indicates that even though some free amino acids can be directly obtained from proteins, these compounds are most probably initially degraded by endopeptidases with the formation of polypeptides which in turn are immediately degraded to the amino acid level by the concerted attack of amino and carboxypeptidases. Some peptides, due to their particular conformation, could conceivably remain adsorbed to the active site of the endopeptidases acting as competitive inhibitors and being responsible for the leveling and decline of the Lowry color in the autolysis experiments. Without the presence of a specific activator it could be expected that the overall degradation of protein would eventually stop. However, the constant synthesis of aminoacyl adenylates for protein synthesis can provide enough endopeptidase activator to maintain and control the overall rate of protein degradation.

The mechanism of activation of the endopeptidase-inhibitor complex can only be suspected at this time. It is possible that the activator molecule, the aspartyl adenylate still adsorbed to the corresponding "activating enzyme" would interact with the endopeptidase-inhibitor complex allowing, through changes in conformation, the release of the inhibitory peptide, which immediately would become degraded by the exopeptidase system.

This scheme predicts a direct relationship between the amount of aspartyl adenylate available at a certain time and the overall

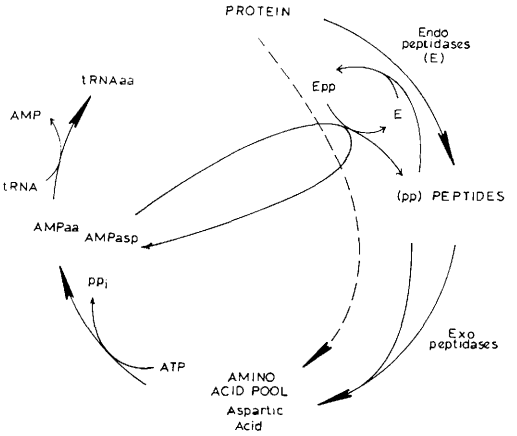

FIG. 7. Scheme representing a working model for the degradation of protein at neutral pH and its possible control by the levels of aspartyl adenylate: Proteins are thought to be degraded to the amino acid level either by a direct attack of the exopeptidase system (minor pathway) or by the concerted attack of endopeptidases and exopeptidases (main pathway). The endopeptidase system becomes inhibited by some of its own products limiting the overall rate of protein degradation. The inactive endopeptidase-inhibitor complex can be reactivated by the interaction of the aspartyl adenylate still adsorbed on the corresponding activating enzyme with the inactive complex. Upon liberation of the inhibitory peptide it immediately becomes degraded by the exopeptidase system allowing the continuous action of the endopeptidases. High levels of aspartyl adenylate would increase the rate of protein degradation and vice versa. This level in turn would depend on the rate of protein synthesis and the availaiblity of "free" aspartic acid.

rate of protein degradation. Therefore, conditions characterized by a high rate of protein synthesis in which the activated amino acids are rapidly used for protein synthesis, as in the case of the infant rats studied by Czajka *et al.* (18), would produce, as described by these authors, a decreased rate of protein catabolism. On the other hand, any potentially destructive accumulation of endopeptidase activator could be easily controlled by the highly effective transaminating system of the liver cell that would limit the supply of aspartic acid available for activation.

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Received March 31, 1971. P.S.E.B.M., 1971, Vol. 138.