Activating Effect of Adenosine-5′-Triphosphate on the Neutral Proteolytic Activity of Liver Homogenates[1] (35172)

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Several investigators have shown that the release of amino acids from kidney, thyroid, or liver slices incubated at neutral pH values (1–3) is greatly inhibited by anaerobiosis or by the presence of inhibitors or uncouplers of the oxidative phosphorylation. Complementary studies have shown that the ability of either intracellular organelles (4) or liver homogenates (5) to degrade denatured albumin is greatly enhanced by the addition of adenosine-5′-triphosphate (ATP).

The fact that these conditions also affect the process of protein synthesis has suggested that protein synthesis and protein breakdown might share one or more reversible steps which could be responsible for the close relationship between these two processes during protein turnover. The purpose of the present work was to further characterize the relationship between ATP and protein degradation and to try to obtain an insight into the possible nature of the intermediary between protein synthesis and protein degradation.

Materials and Methods. Our studies were carried out with two systems. One was an autolyzing system made of a liver homogenate in which the rate of degradation of the endogenous protein was measured by the liberation of Ninhydrin-positive material, and the other one was a modification of the proteolytic assay described by Marks and Lajtha (6) in which the hydrolysis of pepsin-degraded albumin by liver homogenates was determined by the amount of Ninhydrin-positive material liberated under conditions of zero-order kinetics.

The autolytic system consisted of a 20% liver homogenate made in 0.22 *M* phosphate buffer, pH 7.5, containing 0.2% Triton X-100. Samples were pipetted into 25-ml Erlenmeyer flasks and were diluted as follows. The first one (control) was diluted with 10 ml of phosphate buffer, the second one with 10 ml of phosphate buffer containing all the nonessential amino acids.[3] The third was diluted with 10 ml of phosphate buffer containing 1 mg of ATP, a similar amount of this compound being added every 20 min for the duration of the experiment. The last flask received 10 ml of phosphate buffer containing both the mixture of nonessential amino acids and 1 mg of ATP. As in the previous case, a similar amount of ATP was added every 20 min for the duration of the experiment.

To study the effect of ATPase on the rate of autolysis, 2 mg of ATPase (purified Apyrase[4] from potato 3–6 U/mg) were added before the initiation of the incubation. To study the effect of the "amino acid activating enzymes" on the rate of autolysis, 1 ml of a preparation obtained by the method of Hoagland *et al.* (7) containing 1.134 mg of total protein was added to 10 ml of a 20% liver homogenate together with 9 ml of phosphate buffer.

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[3] The amino acid mixture contained 1 mg of each amino acid in 10 ml of 0.22 *M* phosphate buffer, pH 7.5. The nonessential amino acids in the mixture were L-hydroxyproline, L-proline, L-alanine, glycine, L-aspartic acid, L-glutamic acid, L-cystine, L-serine, L-glutamine, L-cysteine, and L-asparagine. The use of a mixture containing all of the essential amino acids did not produce any change in the rate of autolysis.

[4] These compounds and all the L-amino acids were obtained from Sigma Chemical Co., St. Louis, Missouri.

The four flasks were simultaneously incubated at 37° and 2-ml aliquots were taken after 5 min of incubation (zero time) and every 20 min thereafter for 1 hr. The reaction was stopped by pipetting the 2-ml aliquots into test tubes containing 1 ml of 10% trichloracetic acid (TCA). The precipitated protein was removed by centrifugation followed by filtration and the amount of Ninhydrin-positive material in the clear TCA supernatant was determined by the method of Spies (8) using L-tyrosine as standard. The amount of autolysis at a given time was calculated by subtracting the amount of color in the zero-time aliquot from that in the aliquot under consideration. The results were expressed as micromoles of L-tyrosine liberated per gram of liver protein which was determined in the original homogenate according to the method of Lowry *et al.* (9) using bovine serum albumin as standard.

The hydrolysis of denatured albumin was studied with a modification of the system described by Marks and Lajtha (6). Five hundred ml of a 2.4% solution of acid-denatured albumin was digested for 4 hr at 37° with 10 mg of twice recrystallized pepsin[4] after adjusting the pH to 1.5 with HCl. The digested albumin was then heated at 95° for 15 min to denature the pepsin present in solution, and the small amount of precipitate formed was removed by filtration. The clear solution was dialyzed at 4° for 8 hr against three changes of 4.5 liters of 0.22 *M* phosphate buffer, pH 7.5. The final volume was 474 ml and the concentration of protein was 9.75 mg/ml as determined by the method of Lowry *et al.* (9). Gel filtration through Sephadex G-100 of an aliquot of this solution showed the presence of a single peak of protein material of a smaller molecular weight than the original acid-denatured albumin and no evidence of smaller peptides or amino acids.

The assay system contained 0.5 ml of a 4% liver homogenate; 2.0 ml of the solution of pepsin-hydrolyzed albumin; and 6.5 ml of phosphate buffer. When the effect of the amino acid activating enzymes was studied, 0.75 ml of the phosphate buffer was substituted for 0.25 ml of a preparation of "amino acid activating enzymes" containing 1.134 mg of protein/ml as determined by the method of Lowry *et al.* (9), and 0.5 ml of 0.04 *M* $MgCl_2$ containing 1 mg of ATP. The intrinsic proteolytic activity of the "activating enzymes" preparation was determined by incubating 0.25 ml of the preparation in a system similar to the one previously described except that the liver homogenate had been substituted for 0.5 ml of buffer.

The study of the individual effect of each one of the nonessential amino acids was carried out with dialyzed homogenates in order to eliminate the endogenous amino acids. The assay was carried out by substituting 0.5 ml of buffer in the previous system for 0.5 ml of a 1 m*M* solution of the amino acid under study.

The proteolytic activity was assayed as follows. The described mixtures were set in a water bath at 37°. Five min later, 2 ml of the incubation mixture were pipetted into 1 ml of 10% TCA (zero-time aliquot). Fourteen min after this first aliquot, a second one was taken and similarly pipetted into 1 ml of TCA. After removal of the precipitated protein by centrifugation followed by filtration, the amount of Ninhydrin-positive material was determined in the TCA supernatants by the method of Spies (8). The amount of activity was calculated by subtracting the values of the zero-time aliquot from the values of the 14-min aliquot. The activity in the activating enzyme preparation, which in every case was found to be extremely small, was subtracted from the values obtained with the complete experimental mixtures. The results were expressed as micromoles of L-tyrosine liberated per gram of liver protein under the described conditions. The outlined conditions provided a direct relationship between the amount of liver protein present in the system and the amount of L-tyrosine liberated.

Results and Discussion. The rate of release of Ninhydrin-positive material from the endogenous protein in the liver homogenate and the significant activating effect of ATP on this process are presented in Fig. 1. It is interesting to note that the activating effect of ATP could be greatly enhanced by the simultaneous addition of all the nonessential amino acids and that these amino acids alone

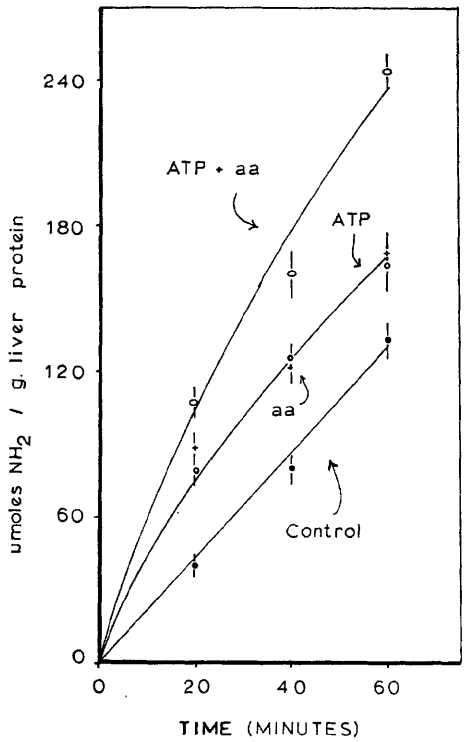

FIG. 1. Effect of a mixture of all the nonessential amino acids, ATP, or both on the rate of autolysis of liver homogenates at neutral pH. Each point corresponds to the average of four independent experiments ± 1 standard error. The NH_2 designation in the ordinate corresponds with the number of micromoles of L-tryosine equivalents liberated during the incubation.

could also increase the rate of autolysis to an extent similar in magnitude to the increase produced by ATP.

These results raised the question as to whether the larger activating effect obtained when ATP was added to homogenates previously enriched with exogenous nonessential amino acids was due to the additive effect of two different proteolytic systems being simultaneously activated or to the enhanced activity of one proteolytic system being activated by a molecular species synthesized from ATP and one or several of the amino acids in the presence of appropriate amounts of an endogenous catalytic system.

The results presented in Fig. 2 showing that the activating effect of the nonessential amino acids can be suppressed by the hydrolysis of the endogenous ATP by added ATPase strongly suggests that the activating effect of the nonessential amino acids and/or of ATP can probably be ascribed to the synthesis of a new molecular species.

One type of hepatic enzyme intimately involved in protein synthesis through the catalysis of the reaction between the L-amino acids and ATP is the "amino acid activating enzymes." These enzymes catalyze the formation of aminoacyl adenylates from free amino acids and ATP in the presence of magnesium, the aminoacyl adenylate formed remaining adsorbed to the enzyme until the aminoacyl moiety is transferred to a specific tRNA molecule. Since liver homogenates normally contain endogenous amino acids and endogenous ATP, it seemed logical to assume that, if any of the aminoacyl adenylates of the nonessential amino acids were to be responsible for the activation of the neutral proteolytic activity, their increased synthesis stimulated by the addition of "amino acid

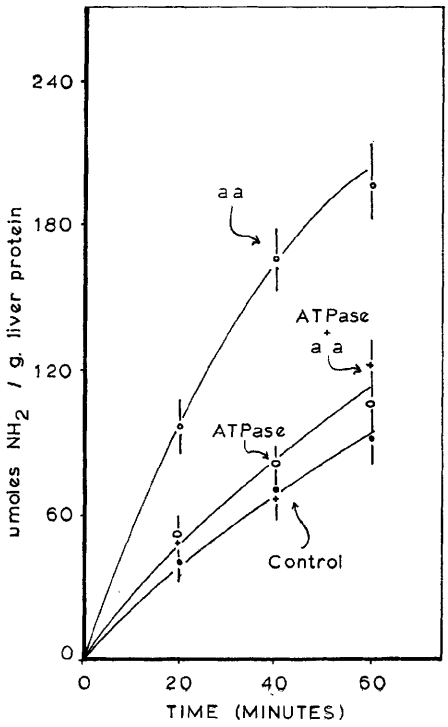

FIG. 2. Effect of ATPase on the ability of a mixture of all the nonessential amino acids to increase the rate of liver autolysis at neutral pH. Each point corresponds to the average of 3 independent experiments ± 1 standard error.

TABLE I. Effect of the Addition of Small Amounts of a Preparation of "Amino Acid Activating Enzymes" in the Presence of ATP and Mg on the Degradation of Pepsin Hydrolyzed Albumin (μmoles of L-tyrosine equivalents liberated/g of liver protein).

	No. of expts.	Mean	SE	Significance level
Control	6	276	20.0	
+ "activating enzymes"	7	386	22.9	0.05
+ "activating enzymes" with a dialyzed homogenate	9	226	12.4	NS

activating enzymes" to the homogenate should result in a measurable increase in the rate of protein degradation. The results of this type of experiment showed a significant increase (60%, $p = 0.05$) in the amount of degradation products found during the first 20 min, this difference progressively decreasing and becoming nonsignificant when the incubation period reached 60 min.

A significant increase in the amount of proteolysis could also be demonstrated when the "amino acid activating enzymes" were added to the proteolytic system containing denatured albumin (Table I). It could be shown that the removal of the endogenous amino acids by dialysis precluded the activation of the proteolytic activity by the "activating enzymes" unless aspartic acid was simultaneously added to the system (Table II). Of all the amino acids present in the mixture of nonessential amino acids, only aspartic acid was found to have activating properties (Table II), the use of a mixture of all the essential amino acids giving negative results.

TABLE II. Effect of Individual Nonessential Amino Acids on the Degradation of Pepsin Digested Albumin in the Presence of ATP, Mg, and "Amino Acid Activating Enzymes" by Dialyzed Homogenates[a] (μmoles L-tyrosine equivalents liberated/g of liver protein).

Amino acid	No. of expts.	Mean	SE
Control	9	226	12.4
+ aspartic acid	5	300	12.7[b]
+ asparagine	2	235	—
+ glutamic acid	5	249	19.4
+ glutamine	5	252	25.1
+ cysteine	5	200	24.8
+ cystine	3	225	—
+ serine	2	230	—
+ proline	3	222	—
+ hydroxyproline	2	253	—
+ alanine	2	252	—
+ glycine	2	216	—

[a] Final concentration of each amino acid in the incubation mixture was 5.6 μg/ml.

[b] $p = 0.02$ with respect to the control.

From the previous results, it seems safe to conclude that the neutral proteolytic activity can be effectively increased by the addition of aspartic acid, alone or in the presence of other nonessential amino acids, ATP, and "amino acid activating enzymes" to liver homogenates, which normally contain all these compounds in concentrations determined by the physiological state of the animal, and that the activating species could be the adenylate derivative of aspartic acid formed by the interaction of the free amino acid with ATP in the presence of the corresponding activating enzymes. These experiments do not allow any conclusions as to the mechanism of activation or to the type of proteases affected; however, they indicate the possible nature of the activator providing an intermediary between protein synthesis and degradation.

Summary. The addition of all the nonessential amino acids in general, and aspartic acid in particular, of ATP, and of small amounts of "amino acid activating enzymes" to liver homogenates produced a definite increase in the rate of protein degradation. The activating effect of the amino acids was shown to be dependent on the presence of ATP and the activating effect of the "amino acid activating enzymes" was shown to be dependent upon the presence of aspartic acid. It was concluded that the activating species may be the aspartyl adeny-

late which would provide an intermediary between protein synthesis and protein degradation.

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