The Apparent Stimulation of Proteolysis by Adenosine Triphosphate in Tissue Homogenates

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Reports that ATP promotes proteolysis in tissue homogenates were reinvestigated. Although ATP increased production of material reacting with ninhydrin or Folin–phenol reagent, ATP did not stimulate protein degradation in such extracts. AMP and adenosine acted similarly to ATP. Deamination of the added nucleotides to produce ammonia caused the increase in ninhydrin-positive material.

Intracellular protein degradation has been recognized as a major factor determining the concentration of individual enzymes as well as the total amount of cellular protein. A number of observations suggest that this process requires metabolic energy. For example, in rat hepatoma cells (Hershko & Tomkins, 1971) inhibitors of energy metabolism suppress the degradation of tyrosine aminotransferase and total protein, and in *Escherichia coli* such inhibitors prevent the selective degradation of abnormal cell proteins and the accelerated degradation of proteins during starvation (Goldberg, 1972; Prouty & Goldberg, 1972). An adequate explanation for this apparent energy requirement is presently lacking. None of the various intracellular or extracellular proteases that have been described in mammalian tissues (Barrett & Dingle, 1971) or bacteria (Matsubara & Feder, 1971) are known to be energy-dependent. Possibly metabolic energy could be required for activating the proteases or for conformational changes in protein substrates, rendering these molecules more susceptible to proteolytic cleavage. Alternatively, if the proteases or their substrates are spatially segregated in the intact cell, energy might be required for transporting the proteins to the site of degradation.

Progress in this area has been limited by an inability to demonstrate this energy requirement in disrupted-cell preparations (Brostrom & Jeffay, 1970). Umana (1970, 1971) and Umana & Feldman (1971) have claimed that increased autolysis occurs at neutral pH in tissue homogenates on the addition of ATP. In most of these studies autolysis was measured in homogenates of liver, cardiac muscle and skeletal muscle of rat by the release into acid-soluble form of ninhydrin-positive material that was assumed to represent α -amino N released from protein. In order to learn more about the possible energy requirements for protein degradation, we have reinvestigated this reported enhancement of proteolysis by ATP in tissue homogenates (Umana, 1970; Umana & Feldman, 1971). We present alternative explanations of these observations.

Tissues from rats (average body wt. 120g) were homogenized in 0.22M-potassium phosphate buffer, pH7.5, as described by Umana (1970). Homo-

genates (5%, w/v) of liver, cardiac muscle and gastrocnemius muscle were incubated in the presence or in the absence of 1 mM-ATP. Proteolysis was measured by the increased production of ninhydrin-positive material that was acid-soluble. As reported previously (Umana, 1970, 1971; Umana & Feldman, 1971), a greater amount of ninhydrin-positive material was found in those tissue homogenates to which ATP had been added (Fig. 1).

To confirm that the ninhydrin assay is a valid measure of proteolysis, protein degradation in these extracts was also measured simultaneously by the release of a specific amino acid, namely tyrosine. This amino acid was chosen because it can be assayed by a highly specific fluorimetric procedure (Waalkes & Udenfriend, 1957) and because it is neither synthesized nor degraded by skeletal muscle (R. Fulks, J. Li & A. L. Goldberg, unpublished work). Although release of tyrosine from protein occurred at significant rates in all extracts, no increase was found on the addition of ATP (Fig. 1). In the liver homogenates, but not in skeletal and cardiac muscle, the amounts of tyrosine actually decreased in the presence of ATP (Fig. 1). Possibly increased protein synthesis, or charging of tRNA, in the presence of ATP could lead to the fall in the amount of released tyrosine. Alternatively the liver contains enzymes for degrading tyrosine, although it is not clear why ATP would enhance tyrosine degradation. In any case, the data on tyrosine release are in sharp contrast with the changes in the amount of ninhydrin-positive material. A number of explanations may possibly account for the contradictory results obtained with the two assays (e.g. ATP may promote production of ninhydrin-positive peptides but not individual amino acids). One possibility is that the ninhydrin assay may not specifically measure protein degradation.

To evaluate proteolysis in an independent manner, a well-characterized substrate, namely ^{125}I -labelled histone, was added to these same homogenates, and its degradation was measured from the release of ^{125}I into acid-soluble form. Although significant hydrolysis of the histone was observed, the addition of ATP was again without effect on this process (Fig. 1). Similar findings were obtained in all three tissue

Fig. 1. Effect of ATP on the release from homogenates of rat heart, liver and gastrocnemius muscle of tyrosine or ninhydrin-positive material into acid-soluble form and on hydrolysis of ^{125}I -labelled histone

Homogenates (5%, w/v) of heart (a), liver (b) and gastrocnemius muscle (c) were prepared in 0.22M-phosphate buffer, pH 7.5, by homogenizing the minced tissues in a motor-driven ground-glass homogenizer (Kontes Glass Co., Vineland, N.J., U.S.A.). The homogenates were strained through 4-ply gauze and incubated at 37°C, with shaking, in the presence (●) or in the absence (×) of 1 mM-ATP. ^{125}I -labelled histone (50 $\mu\text{Ci}/\text{mg}$) was prepared from calf thymus histone (type IIA; Sigma Chemical Co., St. Louis, Mo., U.S.A.) by the method of Hunter (1967). Approx. 0.1 μCi of ^{125}I -labelled histone was added/ml of homogenate for use as a proteolytic substrate. Samples were withdrawn at various times and an equal volume of 30% (w/v) trichloroacetic acid, containing silicotungstate (5%, w/v), was added to each to precipitate tissue proteins. The acid supernatants were assayed for ninhydrin-positive material (Moore & Stein, 1948), with leucine as a standard, and for tyrosine by the fluorimetric method of Waalkes & Udenfriend (1957). Release of ^{125}I was measured by counting the radioactivities of 0.4 ml volumes dissolved in 4 ml of scintillation fluid (Patterson & Greene, 1965) in a Packard Tri-Carb counter. Each point is the mean of three determinations, which agreed within 10%. Control values obtained with non-incubated homogenate were subtracted from each point. All determinations are expressed per mg of protein in the homogenate as determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard.

extracts. The results with ^{125}I -labelled histone and tyrosine suggest that ATP does not in fact enhance proteolysis in such extracts. Further, in experiments with homogenates of gastrocnemius muscle the addition of 1 mM-AMP, -adenosine or -ADP (Table 1, and unpublished work by D. F. Goldspink & A. L. Goldberg) increased the release of ninhydrin-positive material in a similar fashion to ATP. These findings indicate that the apparent stimulation of proteolysis is not dependent on the 'high-energy' bond of ATP and is not related to the energy-dependence of protein catabolism *in vivo*.

We therefore examined the possibility that the released ninhydrin-positive material may not repre-

sent α -amino N from protein. Because ammonia is ninhydrin-positive, we tested whether ATP, AMP or adenosine might stimulate ammonia production in these extracts. Using the Nessler method (Leonard, 1963), we found a much greater production of ammonia in homogenates of gastrocnemius muscle, liver and heart on incubation with ATP, AMP or adenosine (Table 1). In fact, in all of these tissues the increase in ammonia paralleled the apparent increase in α -amino N (Table 1). To determine what contribution the ammonia was making to the ninhydrin assay, a standard curve was constructed relating standard amounts of $(\text{NH}_4)_2\text{SO}_4$ to equivalents of leucine standard used in the ninhydrin assay. When

Table 1. *Effects of ATP, AMP and adenosine on the release from homogenates of rat gastrocnemius muscle, heart and liver of ninhydrin-positive material and ammonia into acid-soluble form*

Homogenates (5%, w/v) of gastrocnemius muscle, heart or liver were incubated in the presence or in the absence of 1 mM-ATP. Alternatively 1 mM-AMP or -adenosine was added to the homogenates of gastrocnemius muscle. Samples were withdrawn at various times and an equal volume of 10% (w/v) HClO_4 was added to each to precipitate tissue proteins. The acid-soluble supernatants were assayed for ninhydrin-positive material (Moore & Stein, 1948) and ammonia (Leonard, 1963), with $(\text{NH}_4)_2\text{SO}_4$ standards in 5% (w/v) HClO_4 . The release of ammonia was expressed in leucine equivalents by use of a standard curve relating the colour developed in the ninhydrin assay on the addition of various amounts of $(\text{NH}_4)_2\text{SO}_4$. All measurements are expressed per mg of homogenate protein. Each value is the mean of three determinations and represents the increment measured on the addition of ATP, AMP or adenosine. Comparison of the first and third columns suggests that the increased production of ammonia can, within experimental error, account for the increments observed with the ninhydrin assay with ATP, AMP or adenosine. (Because of the arithmetic manipulations and possible errors introduced in conversion of units, each value is probably good to 20%.)

Tissue	Ninhydrin-positive material (nmol/mg)		Ammonia production (nmol/mg)		Ammonia expressed as ninhydrin-positive material (nmol of leucine/mg)	
	30min	60min	30min	60min	30min	60min
Gastrocnemius muscle						
Δ ATP	68	116	1	71	4	77
Δ AMP	116	151	97	128	103	133
Δ Adenosine	86	143	128	203	129	206
	15min	30min	15min	30min	15min	30min
Heart						
Δ ATP	28	54	223	456	23	46
Liver						
Δ ATP	12	28	68	370	6	38

the ammonia release was expressed in ninhydrin units, the increased production of ammonia could, within the errors of these experiments, account for the differences in ninhydrin-positive material on the addition of ATP, AMP or adenosine (Table 1, columns 1 and 3).

To determine the source of the ammonia, the neutralized acid supernatants from the muscle homogenates incubated with ATP were analysed by high-pressure liquid chromatography (Brown, 1970). At the beginning of incubation three peaks were recovered from the column, corresponding to AMP (or IMP), ADP and ATP standards. The ATP peak was the largest, because of the addition of exogenous ATP (1 mM). After 60 min of incubation the peak corresponding to AMP or IMP was enlarged, whereas the ADP and ATP peaks had disappeared; 82% of the ADP and ATP lost during the incubation could be accounted for by the increase in the size of the peak corresponding to AMP or IMP. Because the conditions of chromatography used did not separate AMP and IMP, the u.v. spectra of the supernatants were compared before and after incubation. Before incubation the maximum absorbance was found at 257 nm, which corresponds to the maximum absorbance of adenine nucleotides at pH 2. After incubation the maximum absorbance was observed at 247 nm, corre-

sponding to the maximum absorbance of hypoxanthine, inosine or IMP. These findings demonstrate that the exogenous ATP was totally degraded within 60 min to ammonia and hypoxanthine, inosine or IMP.

These results clearly indicate that the increase in ninhydrin-positive material with ATP reflects predominantly an increased production of ammonia, derived from the ATP, rather than an increase in proteolysis. The tissues studied here, particularly skeletal muscle, are known to contain adenylate deaminase (Lowenstein, 1972) and adenosine deaminase (Schmidt, 1955), which can liberate ammonia from ATP and adenosine respectively. In the brain adenylate deaminase has been shown to be activated by the addition of ATP (Stetlow & Lowenstein, 1967). It is therefore possible that the ATP added to the homogenates both activates these deaminases and after dephosphorylation to AMP becomes itself deaminated. Degradation of the added ATP would explain the need for repeated additions of this compound to tissue homogenates in the studies on autolysis reported by Umana (1970). The reported stimulation of proteolysis on the addition of aspartate (Umana, 1970) may also result from increased production of ammonia as a consequence of aspartate's involvement in the purine nucleotide cycle (Tornheim

& Lowenstein, 1972; Lowenstein, 1972). Similarly the present findings can explain the apparent stimulation of protein breakdown on the addition of ribonuclease (Umana, 1971), since treatment of crude homogenates with this enzyme should liberate AMP and thereby ammonia.

Umana (1971) has also reported that in tissue homogenates ATP stimulates proteolysis when assayed by the appearance of material reacting with the Folin-phenol reagent (Lowry *et al.*, 1951). In confirmation of this work, we also found that addition of 1 mM-ATP to a liver homogenate caused a large increase (103%) in Folin-positive material after a 60 min incubation. However, this apparent stimulation of proteolysis by ATP did not involve hydrolysis of a 'high-energy' bond, since an even greater increase (263%) occurred on incubation with 1 mM-AMP. Thus these observations certainly cannot account for the observed energy requirement for proteolysis *in vivo*. The exact nature of this increased Folin-positive material on the addition of ATP or AMP is unclear. The Folin-phenol reaction is thought to measure primarily tyrosine and tryptophan, but the increase in colour induced by ATP occurred under conditions where the concentrations of tyrosine actually decreased (Fig. 1). Lowry *et al.* (1951) have shown that xanthine and uric acid can also react with the Folin-phenol reagent. The increased Folin-positive material in these homogenates may be due to the formation of xanthine or urate from the added ATP or AMP, after dephosphorylation and deamination. The activity of xanthine oxidase is high in liver (De Renzo, 1956), and in muscle homogenates this enzyme has been reported to convert hypoxanthine into xanthine and urate and thereby to increase the intensity of colour on the addition of the Folin-phenol reagent (Willemot *et al.*, 1969). In fact, on this basis Willemot *et al.* (1969) concluded that the presence of purines (e.g. added ATP) in tissue homogenates at neutral and alkaline pH makes the Lowry reaction an invalid measure of protein breakdown.

These data serve as a warning that measurement of the release of material reacting with ninhydrin or even the Folin-phenol reagent is inadequate for investigations of protein breakdown in crude homogenates. Our experiments, and those of others, further indicate that ATP does not directly enhance either acid or neutral protease activities. Although our findings conflict with previous claims (Umana, 1970, 1971; Umana & Feldman, 1971) suggesting that proteolysis in tissue homogenates is stimulated by ATP, they certainly do not exclude the possibility of energy-dependent steps in protein degradation in intact cells or in certain subcellular fractions. The present studies have not investigated possible indirect effects of ATP on the proteolytic process. For example, it has been suggested that ATP is required in the lysosome (a) to maintain an internal acid milieu

(Mego *et al.*, 1972), which is optimal for acid hydrolases, or (b) to facilitate the transport of protein substrates into this organelle (Hayashi *et al.*, 1973). However, the role for the lysosome in normal protein turnover has not yet been clearly demonstrated; in addition, protein breakdown in *E. coli* is also energy-dependent, even though bacterial cells do not contain lysosomes.

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