Reevaluation of the Role of ATP in Autolysis of Liver Protein[1] (37671)

J. E. HUNTER AND A. E. HARPER

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

Evidence for a postulated energy requirement for protein degradation *in vivo* was investigated by measuring the effects of potential energy sources on the release of ninhydrin-positive material during autolysis of rat liver homogenate at pH 7.5. Addition to the reaction mixture of low levels of ATP (9.1×10^{-2} m*M*), an amino acid mixture, or both ATP (9.1×10^{-2} m*M*) plus amino acids all failed to stimulate autolysis of liver proteins as measured by release of ninhydrin-positive material. Large nonphysiological concentrations of ATP (4.8 or 8.5 m*M*) or of 5′-AMP (8.5 m*M*) markedly stimulated the process, however about 80% of the added ATP disappeared within the first 5 min of incubation, whereas ninhydrin-positive material accumulated with time. Measurement of ammonia release during incubation of homogenate with high concentrations of ATP or AMP indicated that after 90 min the amount of ammonia released was proportional to the amount of ATP added from 0–4.8 m*M* ATP and that addition of amino acids to the incubation mixture did not further increase ammonia release. As ammonia increased, so did production of inosine and hypoxanthine. The results indicate that ATP does not enhance proteolysis and provide evidence that ATP is a source of substrate for deamination reactions which contribute ninhydrin-positive material during autolysis of liver.

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Since the 1950's several publications (1–3) have indicated that an energy source such as ATP may be required for intracellular protein degradation in the liver, however the role of an energy source in this process is not clearly established. Recently Brostrom and Jeffay (4) concluded from a study of protein catabolic activity in several types of rat liver preparations (slice, homogenate, and a soluble sonicated preparation), that liver protein catabolism may require a "structural component" (a recognition site, possibly related to interaction of substrate with catabolic enzymes, that would permit specificity in the degradation of proteins) the integrity of which depends upon a supply of metabolic energy, whereas proteolytic activity probably has no such requirement. Haider and Segal (5) have proposed a model in which lysosomes are the sites of intracellular protein degradation and that uptake of protein by lysosomes may be an energy requiring process.

On the other hand, Umaña (6, 7) has suggested that ATP may be involved in the activation of neutral proteolytic activity and has proposed a mechanism for protein degradation involving ATP. He has reported that aspartyl adenylate formed by reaction of aspartic acid and ATP in the presence of an amino acid activating enzyme may be an activator of neutral proteases and an intermediate between protein synthesis and degradation, thus supporting Walter's hypothesis (8) that synthesis and degradation may involve some reversible reactions.

Autolysis provides a way of studying intracellular protein degradation under something approaching physiological conditions since intracellular protein itself is the substrate. We have studied effects of several lev-

els of ATP, a nonessential amino acid mixture, ATP plus amino acids, 5′-AMP, 2′,3′-AMP, cAMP, and adenine on autolysis of protein in liver homogenates. Although we have observed ATP stimulation of the release of ninhydrin-positive material during autolysis our results do not support the concept that proteolysis is activated by ATP. We feel that the autolytic method based on the release of ninhydrin-positive material lacks precision and reproducibility and does not allow reliable measurement of proteolysis.

Materials and Methods. Male rats (250–350 g) obtained from the Holtzman Company, Madison, Wisconsin, were allowed free access to lab chow (Wayne Lab-Blox) and water.

Disodium ATP, 5′-AMP, cAMP, and adenine were obtained from Sigma Chemical Company, St. Louis, Missouri. 2′,3′-Adenylic acid mixed isomer from yeast was obtained from P-L Biochemicals, Inc., Milwaukee, Wisconsin. [2-^{3}H]ATP, 19 Ci/mmole, was obtained from Amersham/Searle Corporation, Arlington Heights, Illinois.

Livers were homogenized in 4 vol of 0.22 *M* potassium phosphate buffer, pH 7.5, containing 0.2% Triton X-100. Autolysis at 37° of a mixture of homogenate diluted with an equal volume of buffer (control) or of buffer containing ATP or amino acids or both was measured by the method of Umaña (6) with minor modifications. Two-ml aliquots of incubation mixture were pipetted into 4 ml of 10% TCA (instead of 1 ml) to eliminate one dilution step prior to ninhydrin assay. The TCA-treated homogenates were centrifuged (26,000*g*, 10 min), and the supernate was diluted 20-fold and assayed for ninhydrin-positive material by the method of Rosen (9) using L-tyrosine as a standard. Protein was assayed by the method of Lowry (10). Results are expressed as μmoles of tyrosine released per g liver protein. $MgCl_2$ (1 m*M*) was used in all experiments with adenine compounds. The presence or absence of $MgCl_2$, however, had no effect on autolysis.

Several levels of ATP were used in these studies. The lower level was that used by Umaña (6): 1 mg ATP (9.1×10^{-2} m*M*) was added at the start of autolysis and the same amount was added every 20 min for the duration of the experiment. The higher level (initial concentration 8.5 m*M* with ATP added only at the start of autolysis) was that shown by Brostrom and Jeffay (4) to inhibit slightly the release of ^{14}C-lysine from labeled protein in 4 hr of incubation at 37°. In later experiments, intermediate levels 2.4 or 4.8 m*M*, with ATP added only at the start of autolysis, were also studied.

In some experiments in which ATP was added prior to autolysis we measured changes in ATP concentration with time. The reaction was terminated by pipetting the incubation mixture into 3.84 ml of 6% perchloric acid (PCA) followed by immediate addition of an equivalent amount (0.16 ml) of 5 *M* K_2CO_3. Supernatant solutions (26,000*g*, 10 min) were analyzed for ATP by the method of Lamprecht and Trautschold (11) and also for ninhydrin-positive material (9). Although some ATP hydrolysis probably occurred on addition of the incubation mixture to 6% PCA (prior to K_2CO_3 addition), hydrolysis was much less than with 10% TCA as the precipitating agent (without subsequent neutralization). Neither acid treatment affected measurement of ninhydrin-positive material released during autolysis.

Ammonia was measured in the TCA supernates colorimetrically by the method of Wergedal and Harper (12) and also by a microdiffusion procedure (13). In the latter method 0.1 ml TCA supernate was added to 0.9 ml water in a liquid scintillation vial and 1.0 ml of 5 *M* K_2CO_3 was added to release ammonia. The vial was immediately stoppered with a No. 2 rubber stopper into which was inserted a glass rod coated with a film of 6 *N* H_2SO_4. The "acidified" rod served as a trap for the ammonia released. Vials were shaken gently for 1 hr after which the "acidified" rods were removed and placed in separate test tubes. One ml of water was added to elute the ammonia; ninhydrin reactions were performed on these samples as before (9) except that the color was developed for 55 min (instead of 15 min) in boiling water. Ninhydrin reactions were also run on neutralized K_2CO_3 solutions remaining in the vials.

The microdiffusion procedure was shown to be specific for ammonia. Negligible am-

monia remained in the K_2CO_3 solutions as verified by colorimetric and by ninhydrin assay. When tyrosine standards were subjected to the microdiffusion procedure, ninhydrin color was detected in K_2CO_3 solutions but not on the "acidified" rods. Hence, total ninhydrin color was assumed to equal the sum of that due to ammonia ("acidified" rods) plus that due to α-amino nitrogen. We reasoned that if proteolysis were stimulated by ATP, we should observe increased ninhydrin-positive material in the K_2CO_3 solutions following the microdiffusion assay for ammonia. Conversely, if deamination were stimulated by ATP, we should observe increased ninhydrin-positive material on the acidified rods but not in the K_2CO_3 solutions.

Purine products of ATP degradation during autolysis were separated by descending paper chromatography. Whole homogenate (100 μliter) was incubated with 100 μliter of [2-^{3}H]ATP (50 μCi/ml, 2.9 mCi/mmole) at 37° for various time periods. The reaction was stopped by adding 200 μliter of 0.25 *M* PCA followed by 10 μliter of 5 *M* KOH. Supernatant solutions (27,700*g*, 10 min) were chromatographed by spotting 40 μliter (followed by 10 μliter of 6 m*M* EDTA) on Whatman #1 chromatography paper and developing in one of the following solvent systems: I) 0.1 *M* phosphate (pH 6.8)/saturated $(NH_4)_2SO_4$/n-propanol, 100/60/2; II) isobutyric acid/concd NH_4OH/H_2O, 66/1/33; III) isopropanol/concd NH_4OH/H_2O, 65/10/25; IV) 95% ETOH/1 *M* $NaC_2H_3O_2$, 7/3; and V) ethyl acetate/formic acid H_2O, 7/2/1. After development spots were detected under a uv lamp, and identified by comparing their positions with those of standards. Radioactive portions of the chromatogram were cut into strips 1-cm wide (starting from the origin) and 1-in. long. The strips were placed in vials, 15 ml of liquid scintillation solution (0.3% PPO, 0.01% POPOP in toluene) were added, and the samples were counted in a Packard Tricarb Liquid Scintillation Spectrometer.

Results. Addition of low levels of ATP (9.1×10^{-2} m*M* initially) or addition of an amino acid mixture (1 mg of each nonessential amino acid) (6) or addition of both ATP and amino acid mixture all failed to stimulate liberation of ninhydrin-positive material (relative to control) during 60 min of incubation (Table I). Similar results were obtained whether these experiments were performed in the presence or absence of 1 m*M* $MgCl_2$. The large standard errors reflect poor reproducibility of this assay.

On the other hand, we observed that large nonphysiological concentrations of ATP (8.5 m*M*), 5′-AMP (8.5 m*M*), or cAMP (8.5 m*M*) in the incubation mixture markedly stimulated (about 4-fold for ATP and 5′-AMP after 60–90 min of incubation; about 2-fold for cAMP) release of ninhydrin-positive material (Fig. 1). Neither 2′,3′-AMP (8.5 m*M*) nor adenine (4.8 m*M*, adenine was insoluble in the incubation medium at a level of 8.5 m*M* at 37°) gave any stimulation. None of these five adenine compounds at 8.5 m*M* contributed significantly to ninhydrin color formation in the colorimetric as-

TABLE I. Effect of ATP and Amino Acids on Autolysis of Liver Homogenate.[a]

Incubation time (min)	Control (8)[b]	1.0 mg ATP (4)	Amino acid (aa) mixture (4)	1.0 mg ATP + aa mixture (3)
		μmoles tyrosine/g protein		
0	0	0	0	0
20	60.3 ± 6.7[c]	61.0 ± 9.4	66.4 ± 7.2	52.7 ± 8.8
40	79.9 ± 8.2	70.0 ± 7.6	92.1 ± 6.7	91.3 ± 7.6
60	91.3 ± 11.6	86.3 ± 13.2	99.5 ± 12.2	114 ± 10

[a] Incubation and assay methods are described in the text.

[b] Number of experiments in parentheses.

[c] ± SEM.

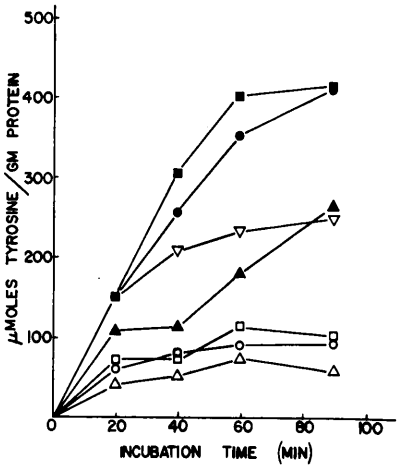

FIG. 1. Effect of adenine compounds on release of ninhydrin-positive material during autolysis of liver homogenate. Adenine compounds were incubated with liver homogenate as described in "Materials and Methods" and ninhydrin-positive material was measured in the TCA supernates. (○), control; (△), 4.8 m*M* adenine (2); (□), 8.5 m*M* 2′3′-AMP (1); (▲), 8.5 m*M* cAMP (1); (●), 8.5 m*M* 5′-AMP (2); (■), 8.5 m*M* ATP (6); (▽), 4.8 m*M* ATP (5). Number of experiments is in parentheses. Standard errors (not shown) were ± 10–18% of experimental points.

say. Also, addition of adenine or ATP up to a final concentration of 5m*M* to control TCA supernates did not enhance ninhydrin color formation. In a prolonged (4-hr) incubation it appeared that after 90 min there was very little further release of ninhydrin-positive material (Fig. 2).

When boiled whole homogenate samples (heated 15 min in boiling water bath) with and without 8.5 m*M* ATP (ATP added after heat treatment) were incubated at 37°, or when whole homogenate with or without 8.5 mM ATP were incubated on ice (0–1°), no significant release of ninhydrin-positive material was observed during 2 hr of incubation. Control samples (whole homogenate with and without 8.5 m*M* ATP) incubated at 37° gave ninhydrin values comparable to those shown in Figs. 1 and 2 indicating that the autolysis procedure was measuring an enzymatic process.

In experiments where initial ATP concentrations were 4.8 or 8.5 m*M*, we found that ATP disappeared very rapidly, about 80% during the 5 min of preincubation at 37°. After 5 min very little ATP was detectable during the remaining autolysis, whereas ninhydrin positive material accumulated with time (Fig. 3). The extent of ATP loss was about the same for either 4.8 or 8.5 m*M* initial levels. We found that the assay was not sufficiently sensitive to detect changes in the concentration of endogenous ATP in control incubation mixtures. Initial ATP concentrations observed at time zero were less than the theoretical values possibly due to slight hydrolysis by the autolysis mixture (on ice) prior to the first sampling or to hydrolysis by 6% PCA prior to neutralization with 5 *M* K_2CO_3.

Results of incubations with high concentrations of ATP (2.4–8.5 m*M*) indicated that after 90 min release of ninhydrin-positive material was proportional to ATP concentration from 0–4.8 m*M* ATP and that ammonia release was stimulated to about the same extent as release of ninhydrin-positive material (Fig. 4). Adenine (4.8 m*M*) stimulated the release of neither ninhydrin-positive material nor ammonia.

Microdiffusion assay of TCA supernates obtained following incubation with 4.8 or 8.5 m*M* ATP or with 8.5 m*M* 5′-AMP indi-

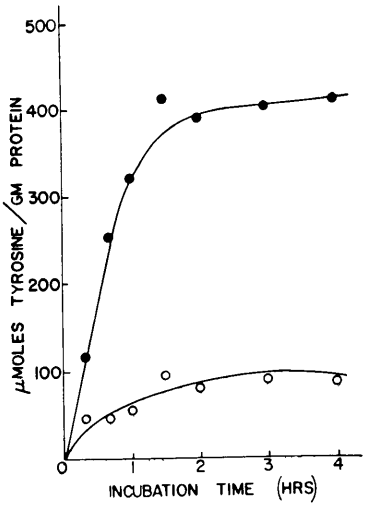

FIG. 2. Effect of 8.5 m*M* ATP on release of ninhydrin-positive material during 4 hr of autolysis of liver homogenate. (○), control; (●), 8.5 m*M* ATP.

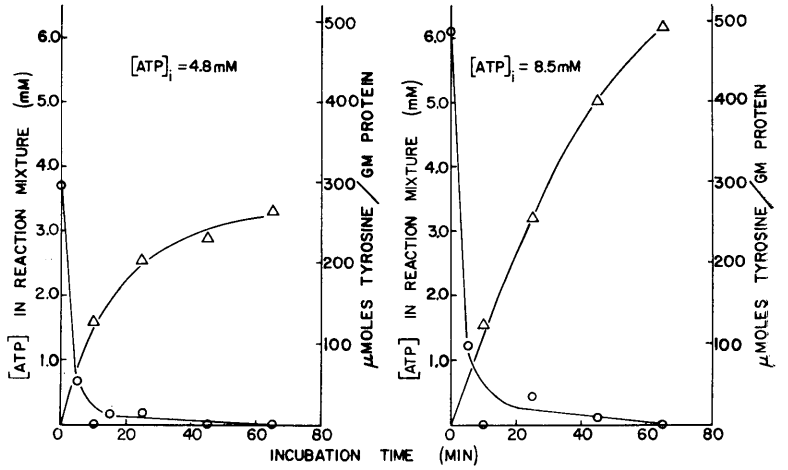

FIG. 3. Changes in concentration of added ATP during autolysis of liver homogenate. ATP, 4.8 or 8.5 mM (○), and ninhydrin-positive material (△) were measured in the same samples.

cated that essentially all of the ninhydrin-positive material detected could be accounted for by ammonia released (Fig. 5). The control and incubation mixtures with 9.1×10^{-2} mM initial ATP concentration gave approximately the same ninhydrin and ammonia assay results. Colorimetric assay of ammonia (12) in the TCA supernates gave ammonia values consistent with those from the microdiffusion assay. Ninhydrin assay of the K_2CO_3 solutions showed wide variability but no stimulation of release of ninhydrin-positive material by ATP which would be expected if ATP stimulated proteolysis.

To test the possibility that ATP might stimulate ammonia release from amino acids during autolysis, we incubated homogenate and 4.8 mM ATP with variable amounts of the nonessential amino acid mixture used by Umaña (6). The results (Fig. 6) indicated that incubation mixtures containing either 3 or 7 ml of amino acid mixture (in 15 ml total

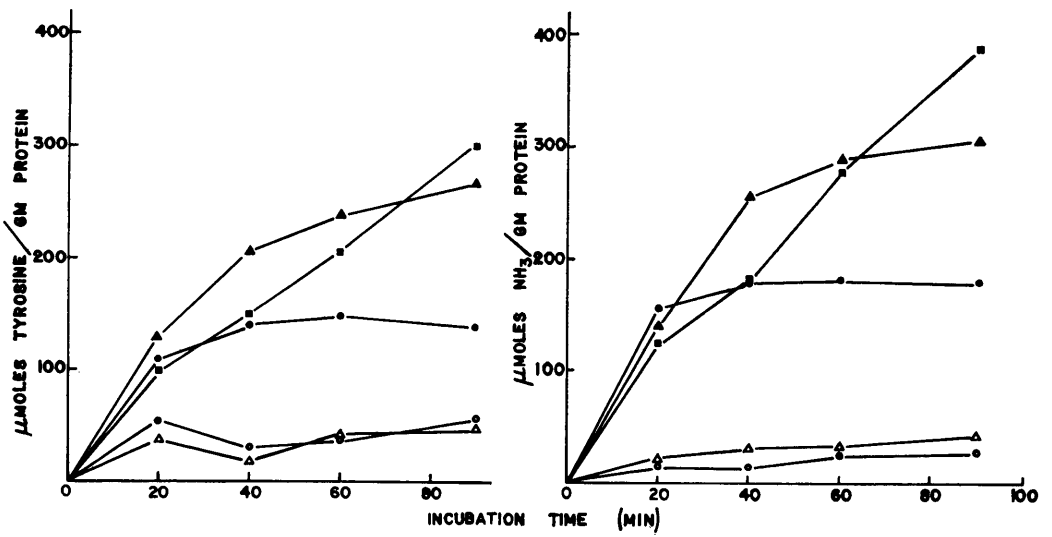

FIG. 4. Effect of ATP concentrations on release of ninhydrin-positive material and ammonia during autolysis of liver homogenate. Ammonia was determined colorimetrically (12). (○), control; (●), 2.4 mM ATP; (▲), 4.8 mM ATP; (■), 8.5 mM ATP; (△), 4.8 mM adenine.

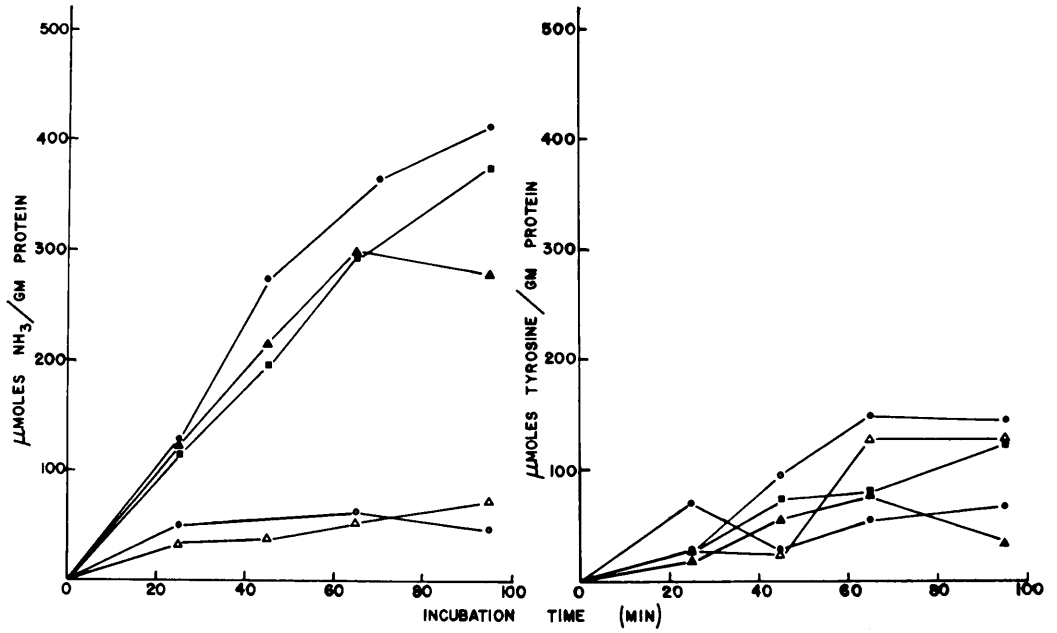

FIG. 5. Effect of ATP and AMP on release of ammonia and α-amino nitrogen during autolysis of liver homogenate. Ammonia was determined by microdiffusion assay (13); α-amino nitrogen was measured by ninhydrin assay performed on the K_2CO_3-neutralized sample following microdiffusion of ammonia. (○), control; (△), 9.1 × 10^{-2} m*M* ATP; (▲), 4.8 m*M* ATP; (■), 8.5 m*M* ATP; (●), 8.5 m*M* 5′-AMP.

vol, thus roughly 2.0 or 4.4 m*M* total in amino acids, respectively) gave the same total ninhydrin color as 4.8 m*M* ATP without amino acids. Seven ml of amino acid mixture without ATP resulted in no net release of ninhydrin-positive material relative to the control. Addition of amino acids plus ATP resulted in about the same ammonia release as did ATP without amino acids, however the ammonia release was not proportional to amino acid concentration (3 ml amino acid mixture plus ATP gave about the same ammonia release as 7-ml amino acid mixture plus ATP) and 7-ml amino acid mixture (4.4 m*M* in total amino acids) gave far less ammonia release than did a nearly equivalent (4.8 m*M*) amount of ATP. Thus deamination of amino acids during autolysis is apparently not as extensive as deamination of ATP. Ninhydrin assay of the K_2CO_3 solutions again showed considerable variability but no clear indication of ATP and/or amino acid stimulation of proteolysis.

Chromatographic analysis of ATP degradation products supported the conclusion that ammonia arose from ATP. Results obtained using system II, which separates most adenine and hypoxanthine nucleotides and nucleosides, indicated that ATP disappeared rapidly (consistent with Fig. 3), that ADP concentration decreased gradually, and that AMP accumulated (Fig. 7A). Some adenosine was formed during autolysis but was no longer detectable at 90 min. Hypoxanthine, a deamination product of adenine, was found to increase with time; however, IMP and inosine, from which hypoxanthine might be released, could not be resolved with this solvent system as they cochromatographed with ATP and AMP, respectively. System I separates ATP and IMP; negligible IMP, however, was detectable; furthermore, neither IDP nor ITP was measurable in systems I or II. System III separates AMP and inosine but with it hypoxanthine and inosine cochromatograph. As shown in Fig. 7B, AMP had disappeared by 90 min and all recoverable radioactivity corresponded with hypoxanthine and inosine. Identical results were obtained with system IV which also separates AMP and inosine but

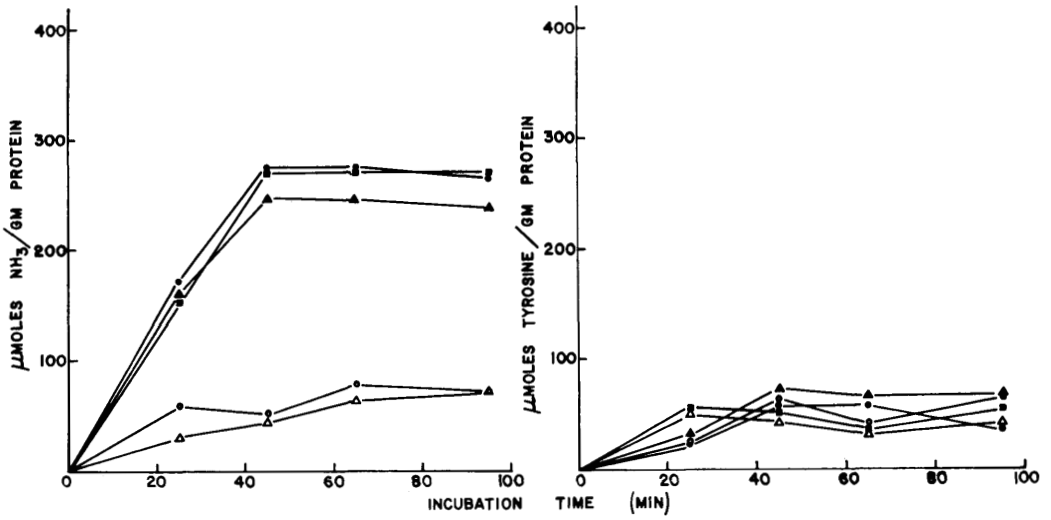

FIG. 6. Effect of amino acids with and without ATP on release of ammonia and α-amino nitrogen during autolysis or liver homogenate. Ammonia and α-amino nitrogen were determined as described for Fig. 5. (○), control; (▲), 4.8 m*M* ATP; (■), 4.8 m*M* ATP + 3 ml amino acid solution (aa); (●), 4.8 m*M* ATP + 7 ml aa; (△), 7 ml aa (no ATP). Total reaction vol = 15 ml.

not inosine and hypoxanthine. Results with system V which separates adenosine and adenine indicated that little if any adenine was formed during autolysis and also confirmed results obtained with systems III and IV. Thus the principal nonvolatile products of deamination appeared to be inosine and hypoxanthine and in Fig. 7A at 90 min these accounted for about 56% and 42%, respectively, of the recoverable radioactivity.

As a control, a heat-treated preparation (500*g*, 10-min supernate of whole homogenate heated at 80°, 10 min) instead of whole homogenate was incubated for 90 min with

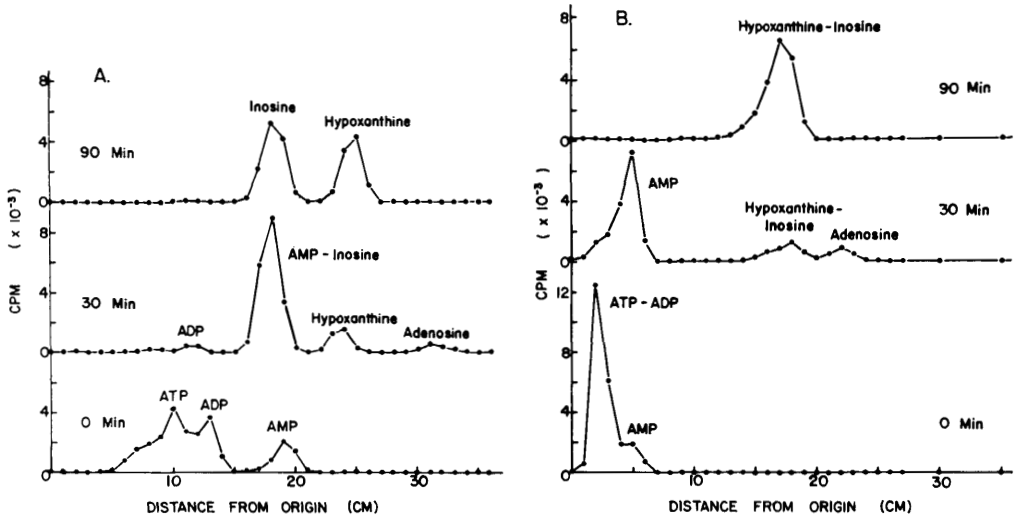

FIG. 7. Paper chromatography of PCA extracts from autolysis of liver homogenate, initial ATP concentration = 8.5 m*M*. (A) System II: isobutyric acid/concd NH_4OH/H_2O, 66/1/33; (B) System III: isopropanol/concd NH_4OH/H_2O, 65/10/25.

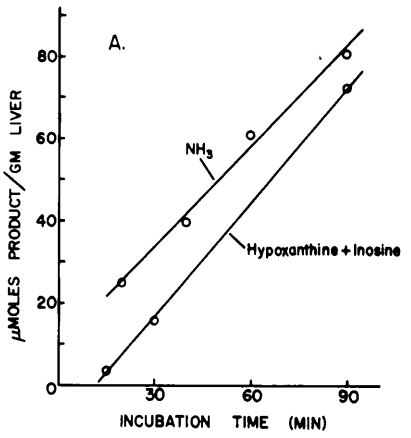

FIG. 8A. Production of ammonia and of hypoxanthine + inosine during autolysis of liver homogenate. Initial ATP concentration = 8.5 m*M*. Data obtained from Figs. 4 and 7B were expressed as μmole product/g liver; slopes were calculated by least squares analysis.

[2-^{3}H]ATP. After development in system II, 92% of recoverable radioactivity was associated with ATP and 8% with ADP; zero time values were the same.

Rates for formation of ammonia and of hypoxanthine plus inosine during autolysis were nearly identical (Fig. 8A) indicating that these products were formed in roughly equimolar concentrations. Some contribution of ammonia from deamination of amino acids may account for the failure of the ammonia curve to pass through the origin. There was apparently a lag period during incubation before hypoxanthine and inosine were produced.

Discussion. Umaña's conclusion that ATP is involved in protein degradation through activation of neutral proteolytic activity was based in large measure on his observations that addition of ATP, a mixture of dispensable amino acids or both to a liver homogenate undergoing autolysis (pH 7.5, 37°) stimulated the release of ninhydrin-positive material. The ninhydrin reaction is sensitive not only to free amino groups of amino acids and peptides but also to ammonia. Our results indicate that ammonia accumulates in liver homogenates undergoing autolysis in the presence of large amounts of ATP and that after 90 min, except at the highest ATP concentration, the amount of ammonia released is proportional to the amount of ATP added (Fig. 8B). This relationship is evident only after maximal autolytic release of ninhydrin-positive material (90 min, Fig. 2). At earlier times (*e.g.*, 20 min) rates of release of ninhydrin-positive material or ammonia were similar and independent of initial ATP concentration (Figs. 4 and 5). ATP does not stimulate the release of ammonia from amino acids (Fig. 6), nor the release of α-amino nitrogen from homogenates (Fig. 5), but products of ATP degradation apparently serve as substrates for deamination reactions.

The occurrence of AMP and adenosine deaminases in animal tissues is well-documented (14, 15) and an ATP-activated AMP deaminase has been isolated and partially purified from 105,000*g* (60 min) supernate of rat liver (16). As ATPase activities are associated with nuclear, mitochondrial and microsomal fractions of rat liver (15), rapid hydrolysis of ATP during autolysis of liver homogenates could result in accumulation of AMP which is subsequently deaminated.

Both 5′-AMP and cAMP, which is probably converted to 5′-AMP by the action of phosphodiesterase, increased the accumulation of ninhydrin-positive material when they were added to the incubation mixture (Fig. 1). 5′-AMP could not be involved in energy-requiring activation of proteolysis but could serve as an effective substrate for deamination reactions. Lack of formation of ninhydrin-positive material when adenine was added

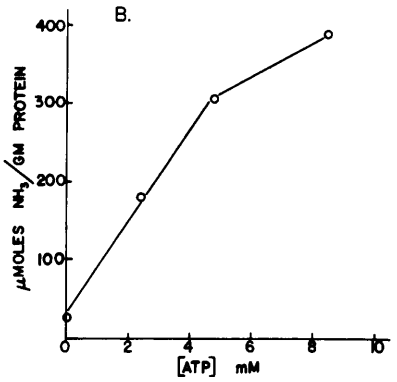

FIG. 8B. Effect of ATP concentration on release of ammonia (colorimetric assay) at 90 min of autolysis of liver homogenate; data from Fig. 4.

to the incubation mixture is consistent with reports that adenine deaminase is absent from animal tissues (14). 2′,3′-AMP is an inhibitor of rat liver AMP deaminase (16) so would not be expected to stimulate release of ninhydrin-positive material.

Accumulation of hypoxanthine and inosine during autolysis (Fig. 7, A and B) and the parallel increases in ammonia and hypoxanthine plus inosine accumulation (Fig. 8A) are consistent with AMP deamination. Although the initial deamination product IMP was not detected, any IMP formed is probably degraded to inosine and ultimately to hypoxanthine in the crude homogenate system. Some AMP was probably converted to adenosine and then deaminated to inosine. Apparent lack of adenine deaminase in our preparations would suggest that hypoxanthine arises primarily from inosine rather than from adenine.

These observations have led us to the conclusion that deamination of ATP or its metabolites rather than proteolysis accounts for most of the increase in ninhydrin-positive material observed when liver homogenates undergo autolysis in the presence of ATP. The lower ATP level used in our experiments (9.1×10^{-2} m*M*) represented an initial concentration of 0.91 μmoles/g of liver; the higher level (8.5 m*M*) was more concentrated than that used by Umaña (0.2 m*M*) but the amounts per g of liver in the two studies were 85 and 91 μmole/g of liver, respectively. These amounts, which were required to stimulate accumulation of ninhydrin-positive material, are about 35 times as great as the normal ATP concentration of 2.5 μmoles/g liver for 150–200 g male rats (11, 17). Further, since ATP is destroyed so rapidly (Figs. 3 and 7), it is unlikely that it could function as an energy donor for the activation of proteolysis *in vitro* over the prolonged period observed in our experiments (Fig. 2). Thus, it seems likely that Umaña's measurements, indicating ATP activation of proteolysis *in vitro,* included ninhydrin-positive material resulting from both proteolysis and deamination. In any study of ATP activation of proteolysis measurement of ammonia arising from deamination of degradation products of ATP must obviously be excluded.

We observed wide variability among ninhydrin results with control and low ATP samples. The variety of enzymatic reactions involving proteases, peptidases, transaminases and deaminases that can occur during autolysis, probably account for the high variability in measurements involving both ammonia and α-amino nitrogen in low activity samples.

Although the possibility of a role for ATP in proteolysis is still unresolved, some recent evidence indicates that ATP may be required for lysosome function. Ignarro *et al.* (18) found that ATP (0.1 m*M*) inhibits release of enzymes from rat liver lysosomes *in vitro* and that this action may be partially mediated by cAMP. Malbica (19) observed that ATP (3 m*M*) stabilized release of acid phosphatase and β-glucuronidase from lysosomes and suggested possible involvement of a lysosomal membrane bound ATPase in controlling acid hydrolase release. Mego *et al.* (20) reported that ATP (0.69–1.25 m*M*, but not ADP, AMP, or cAMP) stimulated proteolytic activity in unbroken mouse kidney and liver heterolysosomes and suggested the existence of an energy-dependent proton pump in heterolysosome membranes which functions to maintain intralysosomal pH in alkaline media. Huisman *et al.* (21) found that lysosomal enzymes can degrade serum albumin to a large extent without addition of ATP, CoA, or thiols and suggested that energy might be necessary for integrity of lysosomes inside the cell or for uptake of proteins into lysosomes, but not for proteolysis proper. Hershko and Tomkins (22) found that ATP depletion prevented inactivation of tyrosine aminotransferase in hepatoma cells in culture and proposed that ATP participates in an early phase of enzyme degradation preceding final proteolysis. Recently Hayashi *et al.* (23) reported that ATP (up to 10 m*M*) facilitates uptake of proteins into lysosomes thereby stimulating proteolysis and suggesting that the ATP concentration of the cytosol might influence the rate of intracellular protein degradation. ATP would not have had such effects in our system since the lysosomes were probably disrupted by homogenizing the liver in phosphate buffer containing Triton X-100. Our results are

more consistent with those of Brostrom and Jeffay (4) who found that in crude systems high ATP concentrations did not enhance release of ^{14}C-lysine from labeled protein.

In discussing mechanisms for control of protein degradation, Schimke (24) suggested that the apparent energy requirement for protein degradation might include a requirement for: a necessary cofactor; removal of degradation products (amino acids and peptides); or maintaining integrity of specific structures such as lysosomes. Furthermore, since the various products of intracellular protein degradation have not been identified, it is conceivable that not all proteins are degraded completely to amino acids; rather, if according to Walter (8) the acceptor of amino acids were tRNA's instead of water, the energy of the peptide bond would be conserved.

Protein degradation is poorly understood at present. Haider and Segal's recent model (5) suggests that the lysosome is the site of intracellular protein breakdown. Schimke (24) believes that the lysosome is important when cell involution or gross changes in the rate of degradation occur (*e.g.*, starvation) but that degradation in the normal steady state involves other system(s) not well defined at present. Recent evidence of Brostrom and Jeffay (25) suggests that nuclear protein catabolism involves both structural and proteolytic components and that RNA may be involved in protein catabolism. It is still not clear whether protein synthesis and degradation share any reversible steps nor how energy is involved in the degradation process.

Note added in proof. While this manuscript was in press similar observations were reported by D. F. Goldspink and A. L. Goldberg, Biochem. J. **134**, 829 (1973).

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