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An Adenosine Triphosphate-Dependent Stabilization of Proteolytic Activity in Heterolysosomes

EVIDENCE FOR A PROTON PUMP

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1. Homogenization of mouse kidneys or livers in 0.25M-sucrose buffered with tris–acetate, pH7.3, resulted in a decreased rate of proteolysis within isolated heterolysosomes containing injected ^{125}I-labelled albumin when these particles were incubated at 35°C. Proteolysis in mouse kidney or liver heterolysosomes isolated from homogenates made in 0.25M-sucrose buffered at pH7.3 was stimulated by pH5 buffer or by additions of ATP. 2. A greater inhibition of proteolysis was produced by including bicarbonate or pH8 borate buffers in the incubation media, and this inhibition was also reversed by ATP. 3. Other nucleoside triphosphates were not as effective as ATP, but GTP and ITP were more effective than CTP or UTP. ADP, AMP, or adenosine 3′:5′-cyclic monophosphate were completely without effect. 4. Although ATP prevented some heterolysosome breakage in media containing bicarbonate, the primary effect appeared to be to promote proteolytic activity. 5. These observations are consistent with the presence of a proton pump in the heterolysosome membrane, which functions to maintain intralysosomal pH in alkaline media.

Formaldehyde-treated, radioiodinated serum albumin is taken up by cells in the liver and kidneys after intravenous injection into mice (Mego & McQueen, 1965; Mego *et al.*, 1967) or rats (Davies *et al.*, 1969). Digestion of the labelled protein proceeds within heterolysosomes formed by the fusion of lysosomes with phagosomes containing the injected material (Straus, 1964; Daems *et al.*, 1969). If the kidneys or livers are removed from animals shortly after injection, these heterolysosomes may be centrifuged from homogenates in cold 0.25M-sucrose. Incubation of the particulate fractions containing heterolysosomes at 35°C results in degradation of the particle-bound labelled protein and the formation of trichloroacetic acid-soluble radioactivity, consisting almost entirely of monoiodotyrosine (Mego *et al.*, 1967). The only requirements for proteolytic activity within mouse liver heterolysosomes during incubation of non-buffered cell-free preparations is an osmotic protective agent such as 0.25M-sucrose and a thiol reductant such as cysteine or 2-mercaptoethanol (Mego, 1971; Mego & McQueen, 1965). Although lysosomal cathepsins require acid pH for activity, the addition of pH5 buffer to heterolysosome suspensions prepared in unbuffered media has no effect on intralysosomal proteolysis. Relatively high concentrations of neutral or alkaline buffers or $NaHCO_3$ partially inhibit intralysosomal digestion, suggesting that these materials enter the heterolysosomes, where they alter the pH, thus inhibiting proteolytic activity. The addition of pH5 buffer to heterolysosomes pre-incubated in $NaHCO_3$ reverses the inhibitory effect of this basic substance (Mego, 1971).

Proteolytic activity in heterolysosomes incubated in the presence of neutral or alkaline buffers suggests that some mechanism may exist in these organelles which functions to maintain internal acidity. In the present study, we describe experiments that suggest that an energy-dependent system exists in the heterolysosome which functions to allow intralysosomal digestive activity to proceed at nearly maximal rates in the presence of alkaline buffers or other basic substances.

Materials and Methods

Bovine serum albumin (crystalline; Mann Research Laboratories, New York, N.Y., U.S.A.) was labelled with carrier-free sodium [^{125}I]iodide by the method of Bocci (1969). Treatment of the radioiodinated protein with formaldehyde was performed as previously described (Mego *et al.*, 1967). The final preparation contained 10mg of protein/ml and about 10^7c.p.m./mg.

Adult Swiss–Webster mice (Southern Animal Farms, Pratville, Ala., U.S.A.) were used in these experiments. The animals were injected intravenously into the tail vein with 0.1–0.2ml of formaldehyde-treated ^{125}I-labelled albumin and they were killed 30min after the injection by removal of tissues under ether anaesthesia. The isolation of kidney and liver heterolysosomes and the assay of proteolytic

activities in these particles were performed essentially as previously described (Mego, 1971; Mego *et al.*, 1967). Tissues were homogenized in cold (4°C) 0.25M-sucrose buffered with 0.025M-tris–acetate buffer, pH7.3. The homogenates were subjected to a preliminary 5min centrifugation at 500*g* in a refrigerated centrifuge to remove any whole cells. The supernatant from this centrifugation was then centrifuged at 30000*g* for 10min, and the resulting pellets were suspended in 12ml of medium consisting of 0.25M-sucrose, 0.05M-2-mercaptoethanol and 1.7mM-$MgCl_2$ unless otherwise indicated. All media were preincubated at 35°C, and incubations were carried out at this temperature.

Heterolysosome breakage during incubation was measured by dilution of 1ml samples of the suspension with 9ml of cold 0.25M-sucrose in 0.02M-tris–acetate buffer, pH7.3. Another 1ml sample was similarly diluted in cold 0.02M-tris–acetate buffer alone. Both dilutions were kept on ice for 10min and then were centrifuged for 10min at 40000*g* to sediment all particulate material. The supernatant fractions were decanted and 4ml samples were counted for radioactivity. The radioactivity in the sample diluted with buffered sucrose was subtracted from that found in the samples diluted with buffer alone, and the results were calculated as percentages of the total radioactivity.

Assays of radioactivity were performed with the Packard scintillation spectrometer and well counter with a 2in thallium-activated sodium iodide crystal. The efficiency of this system was about 50% with a 65keV window bracketing the ^{125}I peaks.

Nucleoside triphosphates and other nucleotides (di- or tri-sodium salts; Sigma Chemical Co., St. Louis, Mo., U.S.A.) were dissolved in 0.25M-sucrose. Additions of these preparations were made to the incubation media in 0.1ml portions immediately after suspension of the particles and then at 10min intervals for a total of four additions unless otherwise specified. In some experiments, the pH values of the mixtures were measured with a Corning pH meter at the termination of incubation to determine pH changes caused by hydrolysis of phosphate bonds.

Results

Homogenization of mouse kidneys or livers in 0.25M-sucrose in 0.025M-tris–acetate buffer, pH7.3, resulted in a decreased rate of proteolytic activity in heterolysosomes containing ^{125}I-labelled albumin compared with rates obtained in these particles isolated by homogenization in sucrose alone. This inhibition was relieved if the particles were suspended in media containing pH5 tris–acetate or acetate buffers or if ATP was added during incubation. ATP produced no further stimulation of proteolysis in the presence of pH5 buffer. Fig. 1 shows these observations in particulate preparations from mouse kidneys. The degree of inhibition of proteolysis in particulate suspensions by homogenization of tissues in the presence of pH7.3 buffer, however, was variable from experiment to experiment. In most cases, the inhibition was approx. 30–35%, but ATP or acid buffer consistently stimulated proteolysis to approximately the same extent. Since $NaHCO_3$ had previously been shown to be an effective inhibitor of proteolysis in mouse liver heterolysosomes (Mego, 1971), further experiments were performed with this substance. Fig. 2 shows the inhibitory effect of $NaHCO_3$ in the incubation medium up to concentrations of 0.1M and the reversal of these effects by ATP in suspensions of kidney heterolysosomes containing injected ^{125}I-labelled albumin. The $NaHCO_3$ produced a greater inhibition of proteolysis and therefore a greater

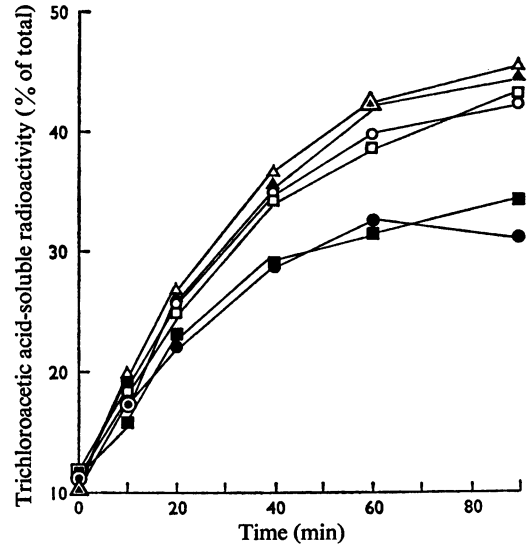

Fig. 1. *Stimulation of proteolytic activity in isolated mouse kidney heterolysosomes by ATP or pH5 buffer*

Kidneys were homogenized in 0.25M-sucrose in 0.025M-tris–acetate buffer, pH7.3, and were centrifuged as described in the Materials and Methods section. Proteolytic activities were measured at 35°C after suspension of the pellets in 0.025M-sucrose with additions as follows: ▲, 0.01M-tris–acetate buffer, pH5.0; △, 0.01M-tris–acetate buffer, pH5.0, and 8.3μmol of ATP; ○, 8.3μmol of ATP; □, 0.01M-tris–acetate buffer, pH7.3, and 8.3μmol of ATP; ■, 0.01M-tris–acetate buffer, pH7.3; ●, no addition. All samples contained sucrose, mercaptoethanol and $MgCl_2$ and the total volume was 12ml. ATP was added in three 0.1ml portions containing 2.76μmol each.

degree of reversal by ATP. Further, the degree of reversal by ATP was the same at all three concentrations of $NaHCO_3$. The pH values of the suspensions shown in Fig. 2 were measured to establish that the effects of ATP were not due to proton formation by hydrolysis of phosphate bonds. The pH changes caused by ATP are shown in Table 1.

Fig. 3 shows the inhibitory effects of 0.025M-$NaHCO_3$ on proteolysis in suspensions of kidney and liver heterolysosomes and the reversal of these effects by ATP and by pH5 buffer. The degree of inhibition by $NaHCO_3$ was greater in kidney particles, and there was a larger stimulation by ATP in these preparations than in liver heterolysosomes.

Sodium citrate and sodium acetate (Fig. 4) as well as $KHCO_3$ (not shown) also inhibited proteolysis in kidney heterolysosome preparations, and this effect was reversed by ATP. These agents raised the pH of the suspensions to 7.5 (citrate) and 6.9 (acetate). Sodium borate and imidazole–HCl buffers, pH8, were also effective inhibitors, but borate buffer was more effective, resulting in a greater degree of reversal by ATP (Fig. 5). Further, borate buffer, pH8, like $NaHCO_3$, produced more consistent effects than did tris–HCl or tris–acetate buffers. The reason for these differences is not known, although permeability may be a factor.

Preliminary experiments showed that the presence of Mg^{2+} ions in the incubation media enhanced the stimulatory effects of ATP. $MgCl_2$ was therefore included in all experiments described above. Mn^{2+} was nearly as effective as Mg^{2+}, but Ca^{2+} ions

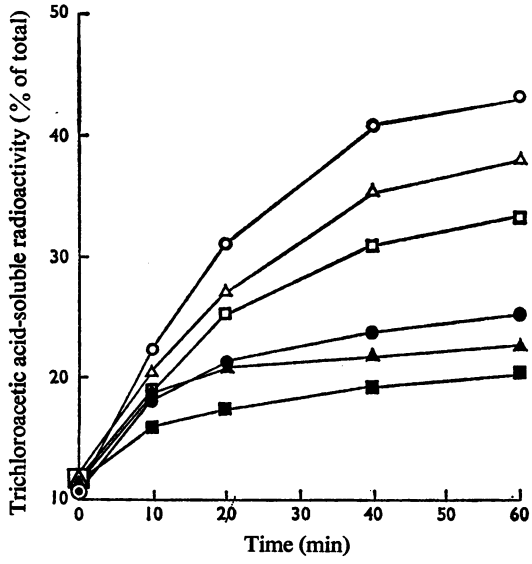

Fig. 2. *Inhibition of intralysosomal digestion of ^{125}I-labelled albumin in isolated mouse kidney heterolysosomes by $NaHCO_3$ and reversal by ATP*

Concentrations of $NaHCO_3$ used were: ○, ●, 0.025M; △, ▲, 0.05M; □, ■, 0.1M. ●, ▲, ■, No ATP; ○, △, □, 15μmol of ATP added in three portions containing 5μmol at 0, 10 and 20min. All samples contained sucrose, mercaptoethanol and $MgCl_2$.

Table 1. *pH changes caused by ATP in mouse kidney heterolysosome preparations incubated with various concentrations of $NaHCO_3$ in* 0.25M-*sucrose*

Concn. of $NaHCO_3$ (M)	pH	
	No ATP	1.3mM-ATP
0.025	8.6	8.2
0.05	8.8	8.6
0.10	9.1	8.9

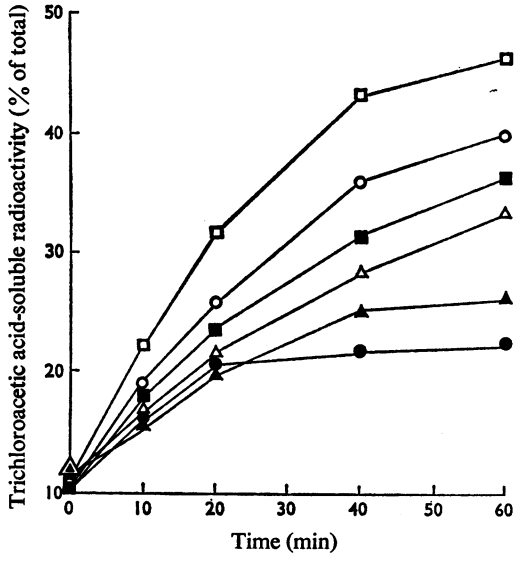

Fig. 3. *Inhibition of protein digestion in mouse liver and kidney heterolysosomes by $NaHCO_3$ and reversal by ATP*

All samples contained sucrose, mercaptoethanol and $MgCl_2$. Additions with liver heterolysosomes were as follows: ▲, 0.025M-$NaHCO_3$; △, 0.025M-$NaHCO_3$ and 20μmol of ATP; ■, 0.025M-tris–acetate buffer, pH5.0. Additions with kidney heterolysosomes were as follows: ●, 0.025M-$NaHCO_3$; ○, 0.025M-$NaHCO_3$ and 20μmol of ATP; □, 0.025M-tris–acetate buffer, pH5.0. ATP was added in four 0.1ml portions containing 5μmol at 0, 10, 20 and 30min.

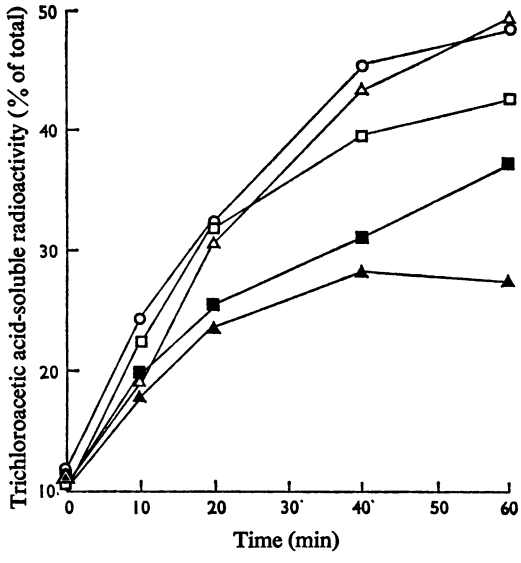

Fig. 4. *Effect of trisodium citrate and sodium acetate on digestion of ^{125}I-labelled albumin in kidney heterolysosomes and reversal by ATP*

All samples contained sucrose, mercaptoethanol and $MgCl_2$. Additions were as follows: ▲, 0.025M-trisodium citrate; ■, 0.025M-sodium acetate; △, 0.025M-trisodium citrate and 20μmol of ATP; □, 0.025M-sodium acetate and 20μmol of ATP; ○, 0.025M-tris–acetate buffer, pH5.0. ATP was added in four portions containing 5μmol each at 0, 10, 20 and 30min.

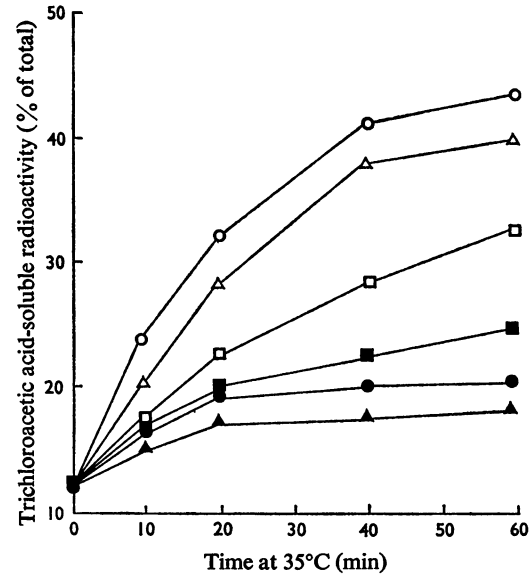

Fig. 5. *Effect of pH8.0 buffers on digestion of ^{125}I-labelled albumin in kidney heterolysosomes and reversal by ATP*

All suspensions contained sucrose, mercaptoethanol and $MgCl_2$. Additions were as follows; ○, ●, 0.025M-sodium borate buffer, pH8.0; □, ■, 0.025M-imidazole–HCl buffer, pH8.0; △, ▲, 0.025M-$NaHCO_3$. ●, ■, ▲, No ATP; ○, □, △, ATP was added in four portions containing 5μmol each at 0, 10, 20 and 30min.

appeared to be inhibitory (Fig. 6). EDTA also inhibited the stimulatory effect of ATP on digestion.

The effects of other nucleoside triphosphates and 3′:5′-cyclic AMP (adenosine 3′:5′-cyclic monophosphate) in reversing the inhibitory effects of $NaHCO_3$ are shown in Fig. 7. ATP was the most effective energy source, but the other purine nucleoside triphosphates tested were partially active. The pyrimidine nucleoside triphosphates were significantly less effective, and 3′:5′-cyclic AMP, ADP and 5′-AMP (not shown) were completely without effect.

Based on the experiments described above, it was not possible to determine if ATP energized some system which functioned to maintain an acid pH within heterolysosomes during digestion, or if this energy source prevented excessive heterolysosome breakage in alkaline media. ATP had no effect on proteolytic activity in heterolysosomes suspended in hypo-osmotic media (0–0.1M-sucrose), nor did it enhance activity in the presence of pH5 buffer (Fig. 1), suggesting that stabilization was not a factor. To determine whether ATP acted primarily as a stabilizer of heterolysosome integrity in alkaline media, breakage of the particles was measured by determining the quantity of particle-associated radioactivity present in heterolysosome suspensions that could be released by osmotic shock. Kidney heterolysosomes were incubated at 35°C in the usual media containing 0.025M-$NaHCO_3$ with or without ATP. The results (Fig. 8) showed that the suspension to which ATP was added contained more labelled protein in osmotically active form, suggesting less heterolysosome disruption. Further, a significantly greater amount of protein digestion occurred in the sample containing ATP, which should result in less undegraded protein in these heterolysosomes. Therefore, if the breakage rate were corrected for digestion, a significantly greater quantity of radioactivity would be present in unbroken heterolysosomes in the suspension containing ATP. These results suggest that the action of ATP may have been to stabilize heterolysosomes. However, if ATP functioned only to retard heterolysosome breakage, then the inhibition of proteolytic activity in the absence of ATP should be proportional to the increased breakage and there should be no in-

hibition of proteolysis if the formation of acid-soluble radioactivity were calculated in terms of the quantity of radioactivity present in intact heterolysosomes at each of the time-intervals in Fig. 8. The results in Fig. 8 therefore were used to calculate rates of digestion in the two suspensions in terms of osmotically-releasable radioactivity rather than total radioactivity. The results (Fig. 9) show clearly that $NaHCO_3$ inhibited digestion in intact heterolysosomes in the absence of ATP. Further, if alkaline conditions inhibited proteolysis by causing heterolysosome breakage, then addition of ATP after digestion had stopped or at a time when the rate had substantially decreased should produce no further stimulation in the rate of digestion. Fig. 10 shows that the addition of ATP at 30min, when digestive activity had ceased, resulted in further proteolysis. ATP therefore stimulated proteolytic activity in unbroken heterolysosomes, an effect which would not be expected if stabilization were the primary function of this energy source.

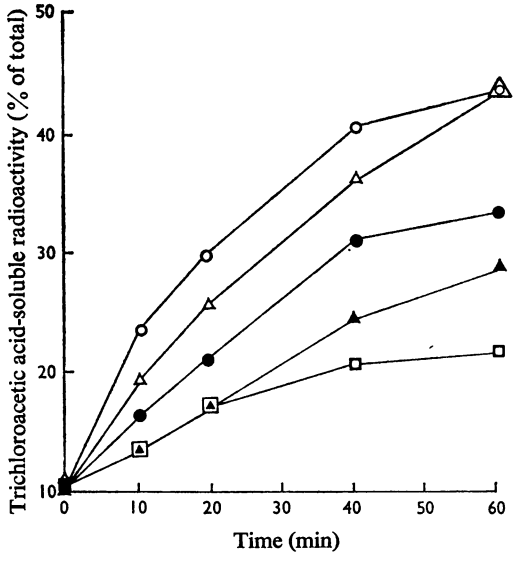

Fig. 6. *Enhancement of the stimulatory effects of ATP on intralysosomal proteolysis at pH8 by Mg^{2+} and Mn^{2+} and inhibition by Ca^{2+} and EDTA*

All samples contained sucrose, mercaptoethanol and 20μmol of ATP added in four portions at 0, 10, 20 and 30min. Additions were as follows: ○, 1.7mM-$MgCl_2$ and 0.025M-sodium borate buffer, pH8.0; △, 1.7mM-$MnCl_2$ and 0.025M-sodium borate buffer, pH8.0; ▲, 1.7mM-$CaCl_2$ and 0.025M-sodium borate buffer, pH8.0; □, 2mM-EDTA and 0.025M-sodium borate buffer, pH8.0; ●, control, no addition.

Discussion

Without direct measurements of pH changes, the evidence that an energy-driven proton pump was responsible for maintenance of proteolytic activity in heterolysosomes under the conditions described in these studies is only circumstantial. However, some mechanism must be present in the heterolysosome membrane, which functions either to prevent the entry of cytoplasmic buffering agents or otherwise to maintain an acid pH within these organelles, since most lysosomal hydrolases have acid pH optima (de Duve, 1963; Tappel, 1969). The cathepsins, in particular, are inactive at neutral or alkaline pH (Mego, 1971; Coffey & de Duve, 1968). Generation of protons during hydrolysis of peptides, phosphate or sulphate esters coupled with an energy-dependent mechanism that prevents the entry of OH^- ions might be sufficient to maintain acidity during intralysosomal hydrolytic activity, but this type of system would perhaps not be sufficient to maintain acid conditions during hydrolysis of basic substances such as histones. Further, the lysosomal membrane is known to be permeable to a variety of substances. These include

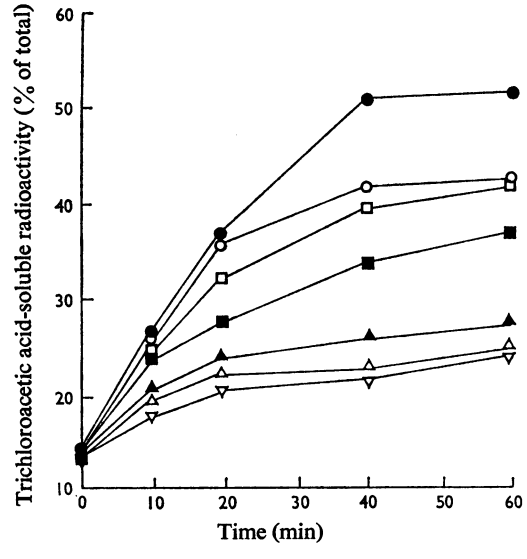

Fig. 7. *Effect of other nucleoside triphosphates and 3′:5′-cyclic AMP on protein digestion in mouse kidney heterolysosomes*

All samples contained sucrose, mercaptoethanol, $MgCl_2$ and 0.025M-$NaHCO_3$. All nucleotides were added in 0.1ml portions (5μmol) at 0, 10, 20 and 30min. ●, ATP; ○, GTP; □, ITP; ■, UTP; ▲, CTP; △, 3′:5′-cyclic AMP; ▽, control, containing no nucleotides.

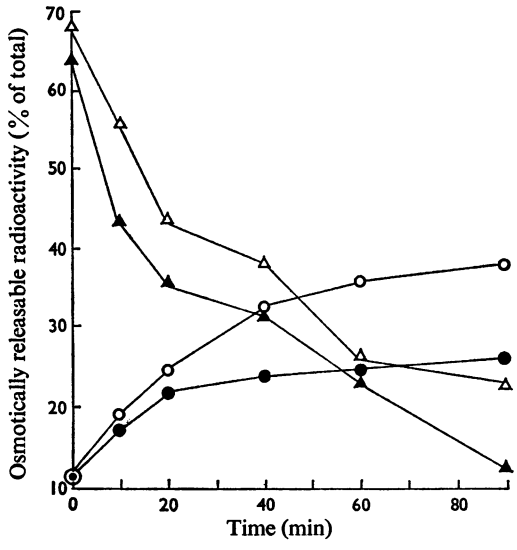

Fig. 8. *Effect of ATP on breakage of mouse kidney heterolysosomes in media containing* 0.025M-$NaHCO_3$ *at* 35°C

Osmotically releasable radioactivity in the presence (△) and absence (▲) of ATP was determined as described in the Materials and Methods section. Digestion in the presence (○) and absence (●) of ATP is shown in the same samples. ATP was added in 0.1ml portions (5μmol) at 0, 10, 20 and 30min.

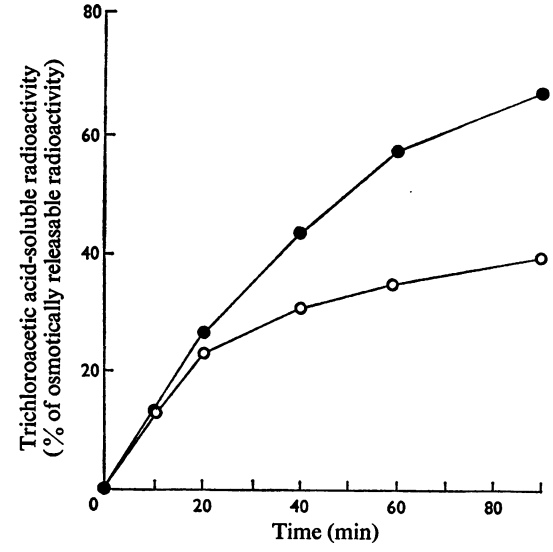

Fig. 9. *Inhibition and stimulation of protoelyticactivity within surviving kidney heterolysosomes*

The heterolysosomes were incubated in a solution containing sucrose, mercaptoethanol, $MgCl_2$ and 0.025M-$NaHCO_3$ with and without ATP. The trichloroacetic acid-soluble radioactivity was calculated as a percentage of the osmotically releasable radioactivity from results in Fig. 8. ●, ATP added; ○, no ATP.

cathepsin inhibitors such as iodoacetamide (Greenbaum & Fruton, 1957; Misaka & Tappel, 1971), which inhibits intralysosomal proteolysis (Mego & McQueen, 1965), and cysteine or mercaptoethanol, which stimulate proteolysis in these organelles. Various carbohydrates, glycitols, amino acids and small peptides (Lloyd, 1969, 1971) also readily penetrate the primary lysosome. Proteolytic activity in heterolysosomes is inhibited by preincubating these particles in media containing bicarbonate (Mego, 1971), and this inhibition is reversed by acid buffers, suggesting that bicarbonate as well as buffer enters the heterolysosome. Lucy (1969) has reviewed other evidence for lysosomal membrane permeability.

Another possible interpretation of the action of ATP reported in the present study is that ATP may promote the transport of labelled amino acids through the heterolysosome membrane. However, ATP stimulated intralysosomal proteolysis only under alkaline conditions and no effects were noted at acid pH. If ATP energized an active transport of degradation products through the membrane, there is no reason to suspect this should not occur under acid conditions.

The evidence in Fig. 8 shows that ATP retarded heterolysosome breakage in alkaline media. The inhibitory effects of bicarbonate could not have been due to excessive heterolysosome breakage, since proteolytic activity was inhibited by bicarbonate in unbroken heterolysosomes (Fig. 9). Further, Fig. 10 shows that ATP promoted renewed proteolytic activity, suggesting that intact heterolysosomes were present but intralysosomal digestion was completely inhibited before addition of ATP. The most logical interpretation of these experiments is that ATP reestablished acidic conditions within the heterolysosomes. The stabilizing effect of ATP noted in Fig. 8 may be similar to that discovered in primary lysosomes by Malbica (1971*a*), in which ATP prevented a thiol-induced release of enzymes. Malbica & Hart (1971) also reported that ATP markedly decreased the release of acid phosphatase and β-glucuronidase from rat liver lysosomes at pH7.4. ATP also prevents breakage of lysosomes by chlorpromazine (Popov, 1966) and *NNN'N'*-tetramethylazoformamide (Malbica, 1971*b*). Duncan (1966) has postulated the presence of an energy-dependent contractile protein in lysosome membranes.

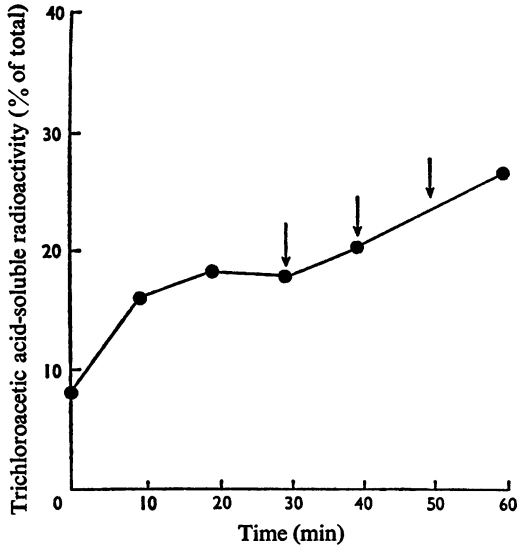

Fig. 10. *Stimulation of the renewal of proteolytic activity in kidney heterolysosomes by ATP after cessation of digestion*

The heterolysosomes were incubated in a medium containing 0.025M-$NaHCO_3$, sucrose, mercaptoethanol and $MgCl_2$. ATP additions (5μmol) are indicated by arrows.

The effect of ATP in the stimulation of intralysosomal proteolysis was considerably more pronounced in kidney preparations than in particles isolated from liver (Fig. 3). The reason for this is not clear at present, although liver preparations contain more particulate material, owing to the larger size of this organ, and there may have been more nonlysosomal ATPase activity in these preparations.

It would be of great interest to determine if some energy-dependent system exists in primary lysosomes which maintains acidity in these particles. Initiation of an ATP-dependent proton pumping system may be a possible mechanism for activation of enzymes in primary lysosomes during cell death, in tissue degeneration during metamorphosis or absorption of tadpole tails, or in other processes in which lysosomes are believed to function. If viruses gain entry into cells through endocytosis, a means for avoiding degradation by lysosomal acid hydrolases might possibly be interference with the action of the proton pump. Further studies in this area may reveal answers to these important questions.

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References

Bocci, V. (1969) *Ital. J. Biochem.* **18**, 346
Coffey, J. W. & de Duve, C. (1968) *J. Biol. Chem.* **243**, 3255
Daems, W. Th., Wisse, E. & Brederoo, P. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), vol. 1, p. 72, North-Holland Publishing Co., Amsterdam
Davies, M., Lloyd, J. B. & Beck, F. (1969) *Science* **163**, 1454
de Duve, C. (1963) *Lysosomes: Ciba Found. Symp.* 1
Duncan, C. J. (1966) *Nature (London)* **210**, 1229
Greenbaum, L. M. & Fruton, J. S. (1957) *J. Biol. Chem.* **226**, 173
Lloyd, J. B. (1969) *Biochem. J.* **115**, 703
Lloyd, J. B. (1971) *Biochem. J.* **121**, 245
Lucy, J. A. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), vol. 2, p. 325, North-Holland Publishing Co., Amsterdam
Malbica, J. O. (1971*a*) *Proc. Soc. Exp. Biol. Med.* **137**, 1140
Malbica, J. O. (1971*b*) *Biochem. Biophys. Res. Commun.* **44**, 1457
Malbica, J. O. & Hart, L. G. (1971) *Biochem. Pharmacol.* **20**, 2017
Mego, J. L. (1971) *Biochem. J.* **122**, 445
Mego, J. L. & McQueen, J. D. (1965) *Biochim. Biophys. Acta* **100**, 136
Mego, J. L., Bertini, F. & McQueen, J. D. (1967) *J. Cell Biol.* **32**, 699
Misaka, E. & Tappel, A. L. (1971) *Comp. Biochem. Physiol.* **38**, 651
Popov, Ch. (1966) *C.R. Acad. Bulg. Sci.* **19**, 1071
Straus, W. (1964) *J. Cell Biol.* **20**, 497
Tappel, A. L. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), vol. 2, p. 209, North-Holland Publishing Co., Amsterdam