The Effect of pH on Cathepsin Activities in Mouse Liver Heterolysosomes

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1. Mouse liver heterolysosomes containing ^{125}I -labelled albumin were incubated at 35°C in 0.25 M-sucrose-0.05 M-mercaptoethanol, and various concentrations of buffers at pH 4, 5, 7 or 8, and the degradation of intraparticulate protein was measured. 2. Buffers at pH 4, 7 or 8 inhibited proteolytic activity and the inhibitions were greater with increasing concentrations of buffers. Tris-acetate buffer, pH 8, was a more effective inhibitor. Tris-acetate buffer, pH 5, neither inhibited nor stimulated proteolytic activity at concentrations up to 0.1 M. 3. Inhibition by pH 8 buffers could be accounted for by increased breakage rates of heterolysosomes. 4. Preincubation of heterolysosomes in 0.2 M-sodium bicarbonate inhibited proteolytic activity when the particles were washed free of bicarbonate, but the inhibition was reversed if these particles were incubated in media containing pH 5 buffer. The inhibition was shown not to be due to an increased breakage of heterolysosomes. 5. It was concluded that the pH within heterolysosomes is about 5, and it is possible to alter reversibly the pH within these particles. The possibility for the existence of an intralysosomal buffering system which maintains the pH at about 5 in heterolysosomes is discussed. This buffering system may be related to the presence of large quantities of acidic lipoproteins in lysosomes observed by other investigators.

Lysosomal hydrolytic enzymes have acid pH optima (de Duve, 1963). Rat liver lysosomal cathepsins degrade proteins most effectively at pH 5, and relatively little or no activity occurs at pH 7-9 (Sawant, Dessai & Tappel, 1964; Coffey & de Duve, 1968; Maunsbach, 1969). A popular assay for cathepsins involves the use of haemoglobin as substrate at pH 3.6 (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). For cathepsins to effectively catalyse protein hydrolysis in heterolysosomes, therefore, the pH within these particles should be acid.

The degradation of proteins may be assayed in heterolysosomes obtained from the livers or kidneys of mice injected with formaldehyde-treated ^{131}I -labelled albumin by measuring the formation of trichloroacetic acid-soluble radioactivity in suspensions of these particles at 35°C (Mego & McQueen, 1965a; Mego, Bertini & McQueen, 1967). The apparent pH optimum for this process is about pH 5, but some activity also is evident at pH 4-9 (Mego & McQueen, 1965b). There appears to be relatively little difference in the rate or extent of proteolytic activity if the particles are incubated at pH 5 or in the presence of 0.01 M buffer, pH 7.4. (Mego *et al.* 1967). Tris-acetate buffer, pH 7, also has little or no more effect on release of labelled

protein from mouse liver heterolysosomes than pH 5 or 6 acetate buffers when these particles are suspended in 0.25 M-sucrose at 22°C (Bertini, Mego & McQueen, 1967). Proteolytic activity is inhibited within heterolysosomes when these particles are incubated in the presence of 1 mM-iodoacetamide (Mego & McQueen, 1965a), a concentration which completely inhibits the activity of cathepsins (Mycek & Fruton, 1956; Greenbaum & Fruton, 1957). The presence of cysteine or some thiol compound is necessary for maximum proteolytic activity in heterolysosomes (Mego & McQueen, 1965a). Iodoacetamide or cysteine, therefore, are apparently able to penetrate the heterolysosome membrane. One possible explanation for the apparent inability of 0.01 M buffer, pH 7.4, to inhibit intralysosomal proteolytic activity is that the pH optimum of cathepsins within intact particles is different. Alternatively, some mechanism may exist within the heterolysosome that is capable of maintaining an acid environment within these particles in the presence of alkaline or neutral buffers, or the membrane is somehow able to prevent the entrance of hydroxyl ions.

In this study, the effects of various concentrations of buffers on intralysosomal proteolytic activity and the capacity of heterolysosomes to degrade

protein in the presence of neutral or alkaline buffers were examined to obtain some information on the buffering system within these particles.

MATERIALS AND METHODS

Crystalline bovine serum albumin (Mann Research Laboratories Inc., New York, N.Y., U.S.A.) was iodinated with sodium [125 I]iodide (Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A.) by the method of Entzian & Dohr (1966) by using chloramine-T. Typically, 140 mg of crystalline bovine serum albumin were dissolved in 5 ml of 0.05M-potassium phosphate buffer, pH 7.2. Carrier-free sodium [125 I]iodide (1.4 mCi in 0.35 ml) was then added, and this was followed by 1.8 ml of freshly prepared chloramine-T (sodium *N*-chloro-*p*-toluenesulphonamide) containing 3.8 mg/ml of water. The mixture was allowed to set at room temperature for 2 h, and was then dialysed for 9 days against 2 l of 0.15M-NaCl and 0.05M-KI with daily changes of the perfusate. On day 9 the medium was changed to 0.15M-NaCl. After 1 week of dialysis, the daily rate of loss of radioactivity from the dialysis bag was less than about 0.5% of the total. The final preparation contained 20 mg of protein/ml and 3400115 c.p.m./mg of protein.

All radioactivity measurements were made with a Packard model 2001 Tri-Carb scintillation spectrometer and well counter with a thallium-activated 2 in crystal of sodium iodide. The efficiency of this system was 24% for 125 I at the 0.035 keV peak with a window of 15 keV. All radioactivity measurements were made with sample volumes of about 4 ml to avoid possible differences due to geometry.

Radioiodinated albumin was treated with formaldehyde as described by Mego *et al.* (1967). In these studies, radioiodinated human serum albumin was treated with formaldehyde because untreated protein was not incorporated into heterolysosomes. Formaldehyde treatment also greatly increased the uptake of bovine serum albumin into mouse liver and kidney particulate fractions (Table 1).

Male Swiss-Webster albino mice (Southern Animal Farms, Prattville, Ala., U.S.A.) weighing about 20–30 g were used in these studies. The animals were injected intravenously into the tail veins, and livers were removed

30 min after injection under ether anaesthesia. Livers were then weighed and homogenized in 20 ml of cold, unbuffered 0.25M-sucrose as described by Mego *et al.* (1967). Homogenates were first centrifuged at 500g for 5 min to remove unbroken cells and other large particles. The supernatants from this centrifugation were divided into the number of samples to be incubated and then centrifuged at 30000g. Unwashed (unless otherwise specified) sedimented particulate material was suspended in 15 ml of the appropriate medium containing 0.25M-sucrose, 0.05M-mercaptoethanol and other additions as indicated. Suspensions were incubated at 35°C, and 2 ml portions were removed at intervals and added to 2 ml of 10% (w/v) trichloroacetic acid. The radioactivity of these samples was counted, they were chilled on ice and centrifuged to sediment precipitated protein; the radioactivity of the supernatants was counted again to determine the percentage of the total radioactivity soluble in the acid. This procedure has been shown to provide a measure of proteolytic activity within intact heterolysosomes and the acid-soluble radioactivity released has been demonstrated to consist of monoiodotyrosine (Mego & McQueen, 1967). This has been confirmed in rat kidney preparations by Maunsbach (1969).

Measurement of heterolysosome breakage during incubation at 35°C was accomplished as described by Mego & McQueen (1967). An alternate method that measured the quantity of intact (trichloroacetic acid-insoluble) labelled protein released from heterolysosomes during incubation was also used. In this procedure, portions of the suspensions were removed at intervals and diluted in 9 vol. of cold 0.25M-sucrose buffered with 0.01M-tris-acetate, pH 7. A 3 ml sample of this dilution was removed immediately and added to 1 ml of trichloroacetic acid (20% w/v) for analysis of total acid-soluble radioactivity as described above. The remaining suspension was centrifuged to sediment all particulate material, and a sample of the resulting supernatant was added to another counting vial containing trichloroacetic acid. After determination of the radioactivity this was centrifuged and the radioactivity of the supernatant was counted again to determine the acid-soluble radioactivity present. The acid-soluble radioactivity was subtracted from the total radioactivity present in the supernatant to obtain a measure of the acid-

Table 1. *Effect of formaldehyde treatment on uptake of 125 I-labelled albumin into particulate fractions of mouse liver and kidney*

Male Swiss albino mice (about 25 g) were injected intravenously with 1 mg of formaldehyde-treated bovine 125 I-labelled albumin or 1 mg of untreated labelled albumin (3400000 c.p.m./mg). Tissues were removed 30 min later under ether anaesthesia (without perfusion) and homogenized as described in the text. Particulate fractions were centrifuged from the homogenates as described, and were washed once in 5 ml of cold 0.25M-sucrose. The radioactivity of samples of the homogenates and the entire washed particulate fractions were counted, and the percentages of the total homogenates contained in the fractions were calculated.

	Untreated		Treated	
	Liver	Kidney	Liver	Kidney
% of injected dose/g of tissue per 20 g mouse	7.0	9.6	12.0	49.0
500g (5 min) particulate fraction, % of total homogenate	5.3	6.3	31.3	23.8
30000g (10 min) particulate fraction, % of total homogenate	5.4	8.2	27.1	28.5

precipitable radioactivity released from the particles into the medium.

Crude mouse liver lysosomal cathepsin was prepared by homogenizing 30 mouse livers in cold 0.25M-sucrose buffered with 0.01M-tris-acetate buffer, pH7.3. This homogenate was centrifuged for 10 min at 39000g to sediment intact lysosomes. The sediment was washed twice by resuspension and centrifugation in a large volume of cold 0.25M-sucrose. The final pellet was suspended in 10 ml of cold water, frozen and thawed several times to break lysosomes and then centrifuged at 40000g for 20 min. The resulting clear light-brown supernatant was dialysed overnight against 1 litre of water at 4°C. Cathepsin activity was assayed by incubation of 0.2 ml of this preparation in a mixture consisting of 0.05M buffer, 0.02M-mercaptoethanol, and 0.5 mg of formaldehyde-treated ^{125}I -labelled albumin as substrate in a total volume of 2.5 ml. Incubations were carried out at 37°C. Samples (0.5 ml) were removed at intervals, added to 2 ml of 10% (w/v) trichloroacetic acid and the radioactivity was counted. They were then chilled, centrifuged, and the radioactivity in the supernatants was counted to determine the percentage of acid-soluble radioactivity produced.

The concentrations of pH 7 or 8 tris-acetate buffers used in this study were with respect to tris. These buffers were prepared by dissolving the appropriate quantity of tris in water and adjusting the pH with acetic acid to 7 or 8 on the pH meter. Tris-acetate buffer, pH 5, was prepared by adding tris to the appropriate quantity of acetic acid.

All centrifugations of particulate fractions were performed in a Sorval RC2-B refrigerated centrifuge by using a SS-34 rotor at 0°C. Sedimentation of trichloroacetic acid-precipitable material was performed at room temperature in an International clinical centrifuge (table model) with a 12-place angle head.

RESULTS

A pH-activity curve for crude mouse liver cathepsin in potassium citrate and potassium phosphate buffers is shown in Fig. 1. Relatively little proteolytic activity occurred at pH 7 or 8. This experiment was also performed with tris-acetate buffers, and the same results were obtained.

Breakage of mouse liver heterolysosomes by the presence of 0.1% (v/v) Triton X-100 or by the absence of sucrose in the media results in inhibition of proteolytic activity (Mego *et al.* 1967). Particle integrity is required, therefore, to demonstrate proteolysis of intralysosomal proteins since breakage results in dilution of enzymes and substrate in the relatively large volume of medium. Inhibition of proteolytic activity in suspensions of mouse liver heterolysosomes may be caused either by breakage or by the entrance of inhibiting agents into the particles.

If the pH for maximum proteolytic activity within heterolysosomes is optimum, the presence of a buffer in the medium should not stimulate the

Fig. 1. Effect of pH on mouse liver cathepsin activity. The concentration of buffer in each sample was 0.05M. Each sample contained 0.5 mg of formaldehyde-treated ^{125}I -labelled bovine albumin as substrate, 2.7 mg of crude cathepsin (0.2 ml) and 0.02M-mercaptoethanol in a volume of 2.5 ml. ●, Citrate buffers; ○, phosphate buffers.

activity further. Fig. 2 shows the results obtained with potassium acetate buffers, pH 4, at concentrations of 0.01, 0.1 and 0.2M on proteolytic activity in mouse liver heterolysosomes. The activity was clearly inhibited by these buffers, and the inhibition was greater at the higher concentrations. At pH 5, however, there was no inhibition of proteolytic activity at buffer concentrations up to 0.1M although there appeared to be some slight inhibition by 0.2M buffer (Fig. 3). This suggests that particle integrity was unaffected at this pH, and the lack of stimulation of proteolytic activity by the presence of pH 5 buffer indicated that the pH was about 5 in the particles. This is consistent with the observation that the optimum pH for hydrolysis of ^{125}I -labelled albumin by lysosomal enzymes is about 5 (Fig. 1). Another possibility is that the presence of pH 5 buffer stimulates proteolytic activity after particle disruption. However, breakage of heterolysosomes containing injected labelled protein by suspensions in media lacking sucrose or containing Triton X-100 also inhibits proteolytic activity under these conditions (Mego *et al.* 1967). Further, Fig. 3 shows that a control sample incubated in the absence of buffer degraded protein at the same rate as the samples containing pH 5 buffer.

At pH 7 (Fig. 4) or pH 8 (Fig. 5) the inhibition of proteolytic activity by higher concentrations of buffers was similar to that noted at pH 4, but pH 8 buffer appeared to be a somewhat more effective

Fig. 2. Effect of potassium acetate buffers, pH4, on proteolytic activity in mouse liver heterolysosomes. Two mice were injected with 0.2 ml (1.5 mg, 4256800 c.p.m.) of formaldehyde-treated ^{125}I -labelled albumin and killed 30 min later. Unwashed 500–30000g liver particulate material was suspended in 15 ml of the appropriate medium and incubated at 35°C. Total radioactivity in each sample was approx. 60 000 c.p.m. The pH of the sample containing no buffer was 6.2. All samples also contained 0.05M-mercaptoethanol. ●, No buffer; ▲, 0.01M-acetate buffer, pH4; △, 0.1M-acetate buffer, pH4; ○, 0.2M-acetate buffer, pH4.

Fig. 3. Effect of tris-acetate buffers, pH5, on proteolytic activity in mouse liver heterolysosomes. Conditions were the same as those described in the legend to Fig. 2 except that tris-acetate buffers, pH5, were used, and each sample contained approx. 71 000 c.p.m. ●, No buffer; ▲, 0.05M-tris-acetate buffer, pH5; △, 0.1M-tris-acetate buffer, pH5; ○, 0.2M-tris-acetate buffer, pH5.

inhibitor. A significant rate of proteolysis occurred in the presence of all concentrations of buffers at pH7 or 8 indicating that a large proportion of the hetero-

lysosomes remained intact. Some inhibition occurred even at a concentration of 0.01M. A higher breakage rate of heterolysosomes at 22°C was caused by 0.05M tris-acetate buffer, pH8, than buffers at pH4–7, but the effect of pH8 buffer was much less than that of tris-acetate buffer, pH9, (Bertini *et al.* 1967).

It is not possible to measure the breakage of heterolysosomes at acid pH by release of intact labelled protein (Mego *et al.* 1967). The released protein becomes bound to particulate material under these conditions (Bertini *et al.* 1967). In experiments on the effect of buffer on release of particle-bound, intact labelled protein, therefore, the controls consisted of particles suspended in media containing 0.01M-tris-acetate buffer, pH7.3. Fig. 6 shows the release of intact labelled protein into the medium in the suspensions incubated in tris-acetate buffers, pH8, shown in Fig. 5. These results indicate that the inhibition of proteolysis by pH8 buffers could be accounted for by an increased breakage of heterolysosomes, and the breakage rates were higher with increasing concentrations of buffer. The experiment illustrated by Fig. 6, however, probably does not represent the true breakage rate since more intact protein would be released by disruption of heterolysosomes in which active proteolytic activity has been inhibited. An increased rate of liberation of intact protein, therefore, may be due to an inhibition of intralysosomal proteolysis and the usual breakage of heterolysosomes observed during the incubation of these particles would result in the release of more protein than in control suspensions.

Experiments on the inhibition of proteolytic activity by neutral or alkaline buffers appear to provide little or no information on the ability of buffers to penetrate the heterolysosomal membrane and to change the internal pH. However, since some proteolysis occurred in the presence of alkaline or neutral buffers, and the particles required at least 20 min for maximum breakage to occur (Fig. 6), it seems reasonable to conclude that some buffer entered the particles but perhaps not in sufficient quantity to inhibit completely proteolytic activity. To test this possibility further, heterolysosomes centrifuged from unbuffered homogenates were pre-incubated for 10 min at 35°C in 0.25M-sucrose containing 0.2M-sodium bicarbonate. Bicarbonate was used because this substance has no buffering capacity at neutral pH, but it should enter the heterolysosome and neutralize the internal pH resulting in an inhibition of proteolytic activity. This inhibition should be reversible if the surviving particles are then washed and incubated in media containing buffer at pH5. A control sample was preincubated in 0.25M-sucrose containing 0.2M-potassium chloride. After preincubation, both

Fig. 4. Effect of tris-acetate buffers, pH 7.3, on proteolytic activity in mouse liver heterolysosomes. Conditions were the same as those described in the legend to Fig. 2 except that each suspension contained 32000 c.p.m. and tris-acetate buffers, pH 7.3, were used. ●, No buffer; ▲, 0.01M-tris-acetate buffer; △, 0.1M-tris-acetate buffer; ○, 0.2M-tris-acetate buffer.

Fig. 6. Effect of tris-acetate buffers, pH 8, on the release of intact (trichloroacetic acid-insoluble) labelled protein from the heterolysosome suspensions in Fig. 5. ●, Control (0.01M-tris-acetate buffer, pH 7.3); ▲, 0.01M-tris-acetate buffer, pH 8; △, 0.05M-tris-acetate buffer, pH 8; ○, 0.1M-tris-acetate buffer, pH 8.

Fig. 5. Effect of tris-acetate buffers, pH 8, on proteolytic activity in mouse liver heterolysosomes. Total radioactivity in each of the 15 ml samples was about 30000 c.p.m. Samples were removed at intervals and analysed for both total acid-soluble radioactivity and for the quantity of acid-insoluble radioactivity (labelled protein) as described in the Materials and Methods section. ●, 0.01M-Tris-acetate buffer, pH 7.3; ▲, 0.01M-tris-acetate buffer, pH 8; △, 0.05M-tris-acetate buffer, pH 8; ○, 0.1M-tris-acetate buffer, pH 8.

samples were diluted with ice-cold 0.25M-sucrose, divided into two equal parts and centrifuged. Particle disruption ceases when heterolysosomes are

chilled to about 4°C even in the presence of high concentrations of pH 8 buffer (J. L. Mego, unpublished work). One of the two parts preincubated in potassium chloride or sodium bicarbonate was suspended in 0.25M-sucrose containing 0.05M-mercaptoethanol, and the other part was suspended in the same medium buffered with 0.05M-tris-acetate buffer, pH 5. The results (Fig. 7) show that the rates of proteolytic activity in the samples preincubated in 0.2M-potassium chloride or transferred to media containing pH 5 buffer were the same. Only the sample preincubated in sodium bicarbonate and transferred to unbuffered medium was inhibited. Samples were also taken to determine the breakage rates of the particles in the suspensions preincubated in potassium chloride or bicarbonate and transferred to unbuffered media. These results are shown in Fig. 8, and they show that breakage rates were approximately the same in these two samples. The sample in which proteolytic activity was inhibited contained more radioactivity that could be released osmotically.

DISCUSSION

Acid pH optima for lysosomal enzymes provide a mechanism for protection of the cell if these enzymes should become released from the lysosome into the cytoplasm. Some mechanism should also exist to maintain an acid pH within heterolysosomes so that

Fig. 7. Reversal of sodium bicarbonate inhibition of intralysosomal proteolysis by pH 5 buffer. Heterolysosomes centrifuged from the liver homogenate of a mouse injected with 2mg (6800000 c.p.m.) of formaldehyde-treated ^{125}I -labelled bovine albumin were preincubated for 10 min in either 0.25M-sucrose containing 0.2M-KCl (—) or 0.25M-sucrose containing 0.2M-sodium bicarbonate (----). The suspensions were then washed and resuspended in media containing 0.25M-sucrose, 0.05M-mercaptoethanol and 0.05M-tris-acetate buffer, pH 5 (▲, △) or unbuffered sucrose-mercaptoethanol (●, ○). Acid-soluble radioactivity was assayed during the course of the incubation as described in the Materials and Methods section. Each suspension was in a volume of 10ml and contained a total of 17800 c.p.m.

Fig. 8. Breakage rates of the heterolysosomes (Fig. 7) preincubated in KCl (●) or NaHCO_3 (○) and then transferred to media without buffer. Samples (1ml) were removed at the times indicated and added to 9ml of cold 0.01M-tris-acetate buffer, pH 7, and to 9ml of cold 0.25M-sucrose containing 0.01M-tris-acetate buffer, pH 7. These suspensions were allowed to set on ice for 10 min and they were then centrifuged. The radioactivity of supernatants was counted and the counts obtained in the samples containing sucrose were subtracted from the corresponding samples containing buffer alone. The results are a measure of the radioactivity that could be released osmotically present in the heterolysosomes at the times indicated.

maximum rates of hydrolytic activities may occur, as well as allow diffusion or transport of degradation products across the lysosomal membrane into the cytoplasm in the presence of cytoplasmic buffering materials. The buffering system within heterolysosomes must also have enough capacity to maintain the pH in the presence of basic degradation products and cytoplasmic buffering components.

In previous studies, we have noted that a considerable quantity of acid-soluble radioactivity is always present in intact heterolysosomes during incubation of these particles at 35 or 37°C (Mego *et al.* 1967). This radioactivity may be as high as 20% or more of the total in the particles (zero-time sample, Fig. 5). However, some of this material may consist of di- or tri-peptides, which are unable to pass through the heterolysosome membrane until they are broken down to smaller units. Lloyd (1969) has studied the penetration of various carbohydrates into primary lysosomes. His studies suggest that some sugars penetrate the lysosomal membrane more readily than others and there appeared to be some correlation with molecular size: disaccharides least readily penetrated the mem-

brane and monosaccharides provided less osmotic protection. There were some exceptions to this generalization, since lactate also protected the lysosomes from osmotic disruption, but glycerol was not effective in this respect. However, all carbohydrates tested, with the possible exception of glycerol, afforded some osmotic protection to the lysosomes. These observations, and the relatively large quantities of cysteine or iodoacetamide required to elicit an effect within heterolysosomes, suggest that substances do not readily enter or leave the heterolysosome through the membrane. Transport through the membrane may be by passive diffusion, although a facilitated diffusion or energized active transport, particularly for maintenance of acid pH within lysosomes, is also a possibility. An adenosine triphosphatase system for maintaining lysosomal integrity has been suggested by Duncan (1966).

From the results obtained in the present paper, it would appear that the existence of an acid pH environment in heterolysosomes is maintained by some internal buffering system rather than by the inhibition of entry of alkaline substances. The suspension of heterolysosomes in media containing

bicarbonate for 10 min at 35°C did not result in disruption of all the particles since active heterolysosomes could be sedimented after this preincubation provided that they were suspended in media containing pH 5 buffer. The presence of pH 5 buffer stimulated proteolytic activity in these particles, suggesting that the internal pH was changed by preincubation in bicarbonate. Since the presence of pH 5 buffer had no effect on heterolysosomes preincubated in potassium chloride or on those that were not preincubated (Fig. 4), the pH was apparently already optimum (about pH 5) in these particles. The inhibition by bicarbonate, neutral or alkaline (pH 8) buffers was never complete, suggesting that a relatively high-capacity buffering system exists in the heterolysosomes. Support for this hypothesis may be found in the discovery of a strongly acidic lipid-like component in lysosomes (Barrett & Dingle, 1967). Lysosomes have also been demonstrated to contain large quantities of acidic lipoproteins (Goldstone, Szabo & Koenig, 1970). These lipoproteins make up about half of the lysosomal protein. Although Barrett & Dingle (1967) and Goldstone, *et al.* (1970) postulate that these acidic components of lysosomes may function to bind lysosomal enzymes, they may also function as acidic buffering systems in heterolysosomes.

Balasubramanian & Deiss (1965) found that bovine thyroid primary lysosomes were more stable at pH 5, although Sawant *et al.* (1964) obtained evidence suggesting that rat liver primary lysosomes exhibit greater stability at pH 6.8–7.2. From the results obtained in the present studies, it is difficult to determine if heterolysosomes are more stable at pH 5 or 7. Although proteolytic activity was inhibited at pH other than 5 and increased quantities of intact labelled protein were released in the presence of pH 8 buffers, this may have been due to the presence of larger quantities of protein in the particles. However, Bertini *et al.* (1967) found that tris-acetate buffer, pH 8, released considerably more labelled protein from heterolysosomes than buffer at pH 7 under conditions in which proteolytic activity was probably greatly diminished (22°C). It seems more likely, therefore, that pH 8 buffer caused an increased breakage rate of heterolysosomes and the inhibition of proteolytic activity observed in the presence of neutral or alkaline buffer in the present study was due to breakage. This breakage was probably not due to a salt effect, since potassium chloride (0.5–1M) will function as an osmotic protective agent for heterolysosomes (Mego *et al.* 1967). Also, the incubation of heterolysosomes containing ¹²⁵I-labelled albumin in 0.5M-potassium chloride and 0.01M-tris-acetate buffer, pH 7.3, causes no greater inhibition of proteolytic activity than 0.25M-sucrose at pH 7.3 (J. L. Mego, unpublished work). Further, the experiments

(Figs. 7 and 8) in which proteolytic activity was inhibited by preincubation of heterolysosomes in 0.2M-sodium bicarbonate show that it is possible to inhibit proteolytic activity by altering the pH within these particles. These experiments also suggest that heterolysosomes were more stable in the absence of buffer, since the breakage rate of bicarbonate-preincubated particles after removal of bicarbonate was approximately the same as that in suspensions preincubated in potassium chloride. (I have also performed experiments to show that bicarbonate induces an increased release of labelled protein from heterolysosome similar to pH 8 buffer.) Therefore, increased breakage of heterolysosomes by alkaline buffers or bicarbonate must somehow be related to the presence of a pH gradient across the lysosome membrane. Since there was little or no inhibition of proteolytic activity in heterolysosomes incubated in the presence of pH 5 buffers, this suggests that these particles were more stable at pH 5.

The possibility has not been excluded that the pH optimum for lysosomal cathepsins is different within the particles than after liberation of these enzymes into the suspending medium. However, inhibition of proteolytic activity by preincubation of heterolysosomes in 0.2M-bicarbonate suggests that although the pH optimum could be broader, the peak of activity still occurs in the acid range. Preincubation in bicarbonate may have raised the pH to a value where a lowered activity occurred but not complete inhibition. A more likely hypothesis, therefore, is that an acidic buffering system exists in heterolysosomes to maintain an acid pH.

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