A Study of Permeability of Lysosomes to Amino Acids and Small Peptides

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(Received 12 August 1970)

The latency of nitrocatechol sulphatase activity was measured in rat liver lysosomes before and after preincubation in 0.25M solutions of five amino acids, three dipeptides and a tripeptide. Rates of increase in 'free' activity were taken as an indication of rates of solute penetration into lysosomes and were correlated with the structure of each molecule studied. In general permeability was greater in solutions of pH 7 than of pH 5 or 6, and dipeptides entered more rapidly than amino acids or triglycine. The conclusions are in good agreement with those obtained by other methods.

The enzymes of rat liver lysosomes can digest susceptible proteins to yield amino acids and small peptides, mainly dipeptides (Coffey & de Duve, 1968), and there is increasing evidence that digestion of endocytosed protein to similar low-molecular-weight products takes place within intact lysosomes *in vivo*. Mego, Bertini & McQueen (1967) isolated liver heterolysosomes from mice injected intravenously with denatured ^{131}I -labelled human serum albumin, and incubated them at 37°C in sucrose-protected buffer. Labelled iodotyrosine was progressively released into the medium, indicating a continuing digestion of protein within the lysosomes. Similar results with lysosomes from rat liver have been reported by Davies, Lloyd & Beck (1971). The digestion of radioiodine-labelled proteins after endocytosis into cells has also been studied in cultured mouse macrophages (Ehrenreich & Cohn, 1967, 1968; Unanue & Askonas, 1968), Sarcoma S180 cells (Gabathuler & Ryser, 1969) and rat visceral yolk-sac (Williams, Lloyd & Beck, 1969). Ehrenreich & Cohn (1968) have also studied the digestion of (^3H)leucine-haemoglobin by macrophages. In each of these experiments, the label appearing in the culture medium was identified as free amino acid, iodotyrosine or leucine, although some of the above authors state that their methods would not have detected small quantities of other products. These results raise the questions as to whether oligopeptides are produced during the course of intralysosomal proteolysis and, if so, whether these could avoid further degradation by escaping through the lysosomal membrane. An

affirmative answer to the first question is suggested by what is known about the substrate specificities of the lysosomal proteinases and peptidases (see Barrett, 1969; Barrett & Dingle, 1970). Proteinase activity is exhibited only by cathepsin D, the possibly related cathepsin E, and a component of cathepsin B (the cathepsin B' described by Otto, 1970). All these enzymes are endopeptidases. Cathepsin A and cathepsin C are exopeptidases, but appear to have no activity towards intact proteins. It is likely therefore that intralysosomal proteolysis results from an initial attack by endoproteinases followed by exopeptidase and endopeptidase degradation of the resulting fragments. Whether such fragments are likely to pass through the lysosomal membrane is the question the present work was planned to answer. The ability of some amino acids, dipeptides and a tripeptide to traverse the lysosomal membrane has been investigated by assessing the degree to which these compounds can afford osmotic protection to isolated rat liver lysosomes. The work is an extension of a similar investigation with carbohydrates (Lloyd, 1969).

METHODS

Measurements of 'free' and total arylsulphatase after incubation of lysosomes in 0.25M solutions of different amino acids and peptides. A centrifugal fraction enriched in lysosomes was prepared from homogenized rat liver, resuspended in 0.25M solution of various amino acids and peptides, and incubated at 25°C. 'Free' and total nitrocatechol sulphatase activity was determined in samples withdrawn from the suspension after 0, 30 and 60 min incubation. Full experimental conditions were described by Lloyd (1969).

The solutions of amino acids and peptides were adjusted

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to pH 5, 6 or 7 by addition of NaOH or HCl. With glutamate and lysine, the final concentration of Na⁺ or Cl⁻ in the incubation media was about 0.25 M, but for all other compounds the concentration of these ions was below 0.01 M, i.e. too low to give any osmotic protection (see Table 2).

RESULTS AND DISCUSSION

Two methods have been available to study the permeability of lysosomal membranes, one biochemical and one cytological. The theoretical basis of the first method, that used in the present investigation, has been discussed in detail elsewhere (de Duve, 1965; Lloyd, 1969). The second method derives from observations on macrophages cultured in media to which various compounds had been added (Cohn & Ehrenreich, 1969; Ehrenreich & Cohn, 1969). Enlargement of lysosomes, visible with the phase-contrast microscope, followed accumulation of certain compounds only, taken to be those not able to pass through the lysosomal membrane and not substrates for any of the lysosomal hydrolases. The accumulated results of these

two approaches in relation to carbohydrates are in such good agreement (see Cohn & Ehrenreich, 1969; Lloyd, 1969) that, although they each give indirect evidence, requiring a number of assumptions, their validity no longer seems in doubt. The remainder of this Discussion will therefore assume a correlation between the ability of a compound to afford osmotic protection to lysosomes and its inability to pass the lysosomal membrane.

Table 1 shows that preincubation of lysosomes in 0.25 M solutions of various amino acids and peptides increases the availability of arylsulphatase to its substrate. The extent of the increase is, however, very dependent on the nature of the preincubation solution. In general, solutions of dipeptides afforded less osmotic protection than those of amino acids or of the tripeptide. Table 1 also shows that the pH of the medium is an important factor determining penetration of amino acids and small peptides into lysosomes. With the exception of glycine and alanine, all the solutes entered considerably more rapidly at pH 7 than at pH 5 or 6. This effect may be due to some intrinsic property of the lysosomal

Table 1. 'Free' nitrocatechol sulphatase of rat liver lysosomes preincubated at 25°C in various media

Each value (mean $\pm$ s.d. of three experiments) is expressed as a percentage of total activity.

Medium (0.25 M)	pH	Preincubation	...	Activity (% of total activity)		
				0 min	30 min	60 min
Glycine	5			8.0 $\pm$ 0.5	11.4 $\pm$ 1.6	18.7 $\pm$ 1.4
	6			6.3 $\pm$ 0.9	7.3 $\pm$ 0.6	12.1 $\pm$ 1.3
	7			8.8 $\pm$ 1.3	10.8 $\pm$ 0.6	16.4 $\pm$ 2.0
L-Alanine	5			5.6 $\pm$ 0.5	7.9 $\pm$ 1.3	11.5 $\pm$ 1.5
	6			7.0 $\pm$ 2.4	6.5 $\pm$ 2.4	11.3 $\pm$ 1.1
	7			5.1 $\pm$ 1.4	5.5 $\pm$ 1.1	7.9 $\pm$ 1.9
L-Valine	5			6.1 $\pm$ 1.1	9.0 $\pm$ 1.4	21.6 $\pm$ 3.3
	6			7.2 $\pm$ 1.4	9.3 $\pm$ 0.9	20.7 $\pm$ 1.2
	7			5.6 $\pm$ 0.7	10.7 $\pm$ 3.6	34.0 $\pm$ 4.9
L-Glutamate	5*			6.1 $\pm$ 1.9	9.5 $\pm$ 1.5	14.9 $\pm$ 1.9
	6			6.8 $\pm$ 3.0	9.1 $\pm$ 3.4	12.0 $\pm$ 2.2
	7			5.5 $\pm$ 0.8	22.2 $\pm$ 7.2	32.6 $\pm$ 5.9
L-Lysine	5			7.2 $\pm$ 0.3	10.8 $\pm$ 1.1	14.4 $\pm$ 3.5
	6			5.1 $\pm$ 1.2	8.8 $\pm$ 2.2	12.6 $\pm$ 4.1
	7			7.3 $\pm$ 0.9	15.7 $\pm$ 4.0	29.8 $\pm$ 10.3
Glycylglycine	5			7.2 $\pm$ 0.6	32.4 $\pm$ 6.8	55.2 $\pm$ 8.9
	6			5.6 $\pm$ 1.1	28.8 $\pm$ 2.8	53.8 $\pm$ 5.4
	7			8.1 $\pm$ 1.2	64.7 $\pm$ 3.6	86.1 $\pm$ 2.5
Glycyl-L-alanine	5			7.2 $\pm$ 1.8	16.0 $\pm$ 4.1	23.7 $\pm$ 6.7
	6			6.2 $\pm$ 0.9	19.8 $\pm$ 2.2	28.5 $\pm$ 4.9
	7			6.8 $\pm$ 0.3	47.0 $\pm$ 1.3	63.5 $\pm$ 1.1
Glycyl-L-valine	5			7.1 $\pm$ 1.0	15.6 $\pm$ 2.0	24.4 $\pm$ 3.9
	6			7.3 $\pm$ 2.1	13.2 $\pm$ 0.2	18.6 $\pm$ 1.5
	7			6.3 $\pm$ 0.3	30.3 $\pm$ 8.8	59.3 $\pm$ 2.9
Glycylglycylglycine	5			6.0 $\pm$ 0.6	11.3 $\pm$ 1.3	16.4 $\pm$ 2.2
	6			5.5 $\pm$ 0.3	11.5 $\pm$ 0.6	14.6 $\pm$ 2.4
	7			6.6 $\pm$ 0.7	15.9 $\pm$ 3.1	25.2 $\pm$ 4.5

* Results refer to five experiments.

Table 2. 'Free' nitro catechol sulphatase of rat liver lysosomes preincubated in NaCl solutions

Each value is expressed as a percentage of total activity.

Concn. of NaCl (M)	Preincubation ...	Activity (% of total activity)		
		0 min	30 min	60 min
0.01		93.1	91.8	95.5
0.05		57.5	66.7	72.5
0.10		11.9	13.4	16.0
0.25		6.1	6.6	8.8

membrane, or alternatively to pH-dependent changes in the ionic species of the molecules present. That the effect of pH is not the same for all amino acids tends to argue against the first of these explanations. So does the observation that the degree of osmotic protection afforded by mannitol is not affected by changes of pH within the range 5–7 (J. B. Lloyd, unpublished work).

The five amino acids studied were those soluble up to 0.25M in water at 25°C. Each provided osmotic protection considerably better than that given by glucose (Lloyd, 1969). The smallest molecules, glycine and alanine, were the most efficient, and their effect was largely unaffected by pH over the range studied. These compounds have molecular weights below 100 and the apparent difficulty they experience in passing through the membrane is doubtless related to their charged nature. Previous studies have shown that anions such as β -glycerophosphate, gluconate and glucuronate enter lysosomes much more slowly than uncharged molecules of similar size (Appelmans & de Duve, 1955; Lloyd, 1969). Valine enters lysosomes more rapidly than glycine or alanine and this may be related to increased hydrophobicity due to the isopropyl side chain. Owing to the presence of 0.25M-Cl⁻ or -Na⁺ in the solutions of lysine and glutamate, the results with these two compounds must be compared with those on 0.25M-sodium chloride. Table 2 shows the extent to which sodium chloride can protect lysosomes in this system. Predictably concentrations below 0.1M fail to protect, and the results with hyperosmotic (0.25M) and hyposmotic (0.1M) sodium chloride indicate a relatively slow passage of the ions through the lysosomal membrane under the experimental conditions used. Comparison between 0.25M-glutamate, 0.25M-lysine (Table 1) and 0.25M-sodium chloride (Table 2) suggests that glutamate passes the lysosomal membrane more rapidly than Cl⁻ and lysine more rapidly than Na⁺.

The three dipeptides all pass the lysosomal membrane rapidly. At pH 7 passage is particularly rapid. Glycylglycine (mol.wt. 132) enters con-

siderably more quickly than valine (mol.wt. 117). Glycylalanine enters more quickly than either glycine or alanine, but slower than glycylglycine. Glycylvaline behaves like valine at pH 5 or 6, but enters at a higher rate at pH 7. A clue to the rapid penetration of dipeptides, as compared with amino acids, may be found in the results of conformational analysis. Dipeptides can exist at rotamers in which the positive and negative charges are *cis*, and these will be the favoured conformations (Roberts & Jardetzky, 1970). Such 'charge neutralization' might lead to a molecular species able to penetrate more easily into the membrane's hydrophobic phase. The somewhat slower penetration of glycylalanine and glycylvaline may reflect steric hindrance in the formation of *cis* rotamers or simply a retardation due to the increase in molecular weight. The passage of dipeptides at pH 7 is particularly rapid, and it may be relevant that glycyl dipeptides have pK_a (NH₂) approx. 8.2 (cf. glycine 9.7), so that the formation of unprotonated species at pH 7 will be greater. Triglycine (mol.wt. 189) enters less rapidly than the slightly smaller glycylvaline (mol.wt. 174), particularly at pH 5 and 7. This may be because molecular size becomes the critical factor around this value or may be a further indication of some special feature peculiar to dipeptides, e.g. the 'charge neutralization' discussed above. A further indication that penetration into lysosomes reflects both molecular weight and, in some way, 'hydrophobicity', is given by considering the melting points of glycine, diglycine and triglycine. Glycine has the highest melting point (292°C) and penetrates slowest; this is followed, in both ease of penetration and lower melting point, by the tripeptide (m.p. 244–246°C) and then the dipeptide (m.p. 215–220°C).

Ehrenreich & Cohn (1969) tested a series of small peptides (mol.wt. 160–294) for ability to induce vacuolation in cultured mouse macrophages. Only two compounds were effective, D-alanyl-D-alanyl-D-alanine and D-glutamyl-D-glutamic acid. With the exception of one dipeptide, D-arginyl-D-valine, which was cytotoxic, the ineffective molecules were

either susceptible to hydrolysis by macrophage cell lysates (or by enzymes in the culture medium) or had molecular weights below 200. In the latter group were five dipeptides (mol.wt. 160–190), each containing D-amino acids and resistant to enzymic hydrolysis, and Ehrenreich & Cohn's (1969) conclusion that these can pass through the lysosome membrane is consistent with the results of the present study. Their concept of a barrier to molecules of mol.wt. greater than 200 is not negated by the present results, but the apparent ease with which iodotyrosine escapes from lysosomes is evidence that parameters other than molecular size are important. Ehrenreich & Cohn (1969) did not investigate the effects of amino acids but, since their method detects only the non-penetrating solutes, it would not have revealed the relative difficulty with which some amino acids apparently pass the lysosomal membrane.

The results presented here support the concept that some amino acids and small peptides can pass through the lysosome membrane. The largest molecule tested, triglycine, did not pass rapidly, a result consistent with a hypothesis that the membrane is impermeable to larger peptides. Thus the properties of the lysosomal membrane may increase the efficiency of the cell's digestive apparatus, by ensuring that the initial products of protein degradation do not escape from the vacuolar system but are retained for further attack by lysosomal enzymes. The finding that some dipeptides can pass through the lysosomal membrane much more rapidly than can their constituent amino acids suggests that some dipeptides are probably released from lysosomes during intralysosomal proteolysis *in vivo*. This suggestion is supported by observations that the enzymes of rat liver lysosomes lack the ability to cleave certain oligopeptides, mainly dipeptides, and that suitable peptidases are found only in the cytosol (Coffey & de Duve, 1968).

I thank the Florida Division of the American Cancer Society for a grant and Mrs Gwenda Denner for excellent technical assistance. The work was carried out during the tenure of a Wellcome Travelling Fellowship, and I thank Dr W. J. Whelan for the hospitality of his Department and for his interest in the work. I also thank Dr M. L. Games for some helpful discussions of the results.

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