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RUPTURE OF RAT LIVER LYSOSOMES MEDIATED BY L-AMINO ACID ESTERS

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SUMMARY

Treatment of rat liver lysosome suspensions with 0.5–20 mM α-L-amino acid esters results in a progressive loss of latency of lysosomal enzyme activity. The increase in available acid phosphatase activity in lysosomal suspensions is correlated with the decrease of turbidity of these suspensions. Ester mediated turbidity decrease is dependent upon ester concentration, and the pH and ionic strength of the suspending medium. D-Stereoisomers of amino acid esters do not exhibit comparable capacity to damage lysosomes.

α-L-Amino acid esters were found to be substrates for neutral lysosomal esterase and transpeptidase activity. The D-stereoisomers are degraded at much lower rates. These data support the hypothesis that ester dependent lysosomal rupture is mediated by the specific interaction of the ester with a structural or functional lysosomal protein.

INTRODUCTION

Lysosomes are membrane-limited cytoplasmic particles which contain many hydrolytic enzymes. *In vitro* the latency of the activity of these hydrolases is taken as a measure of the degree of integrity of a lysosomal population. Loss of latency, or activation of the intralysosomal enzymes, occurs either simultaneously with or immediately prior to the release of enzymes to the extralysosomal space[1,2]. Organization and control of such structure-linked latency is a fundamental function of the regulation of lysosomal activity in cells. Although many studies on the stability of lysosomes *in vivo* and *in vitro* have been carried out, the mechanism of initiation of lysosomal damage is still not fully understood.

Rupture of lysosomes as a result of changes in the osmotic pressure balance between the intra and extra lysosomal space has been studied in some detail. Two situations leading to osmotic burst of the vesicles have been described. (a) A hypoosmolar suspending medium[3]. (b) A medium containing an isoosmolar concentration

Abbreviations: ATEE, acetyl-L-tyrosine ethyl ester; BAEE, benzoyl-L-arginine ethyl ester; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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of a permeable solute[4,5]. In both cases no interaction of the solute with intralysosomal constituents was implied.

This communication presents evidence that α-L-amino acid esters damage lysosomal integrity when present in an osmotically protected lysosomal suspension at a concentration of 0.5–20 mM. Several of the parameters affecting the rupture by these compounds and their specificity have been studied. The reaction pattern of a lysosomal enzyme composite on several D- and L-amino acid esters has been investigated.

MATERIALS AND METHODS

L- and D-alanine methyl ester, L- and D-phenylalanine methyl ester, L- and D-tyrosine methyl ester, glycine methyl ester, acetyl-L-tyrosine ethyl ester (ATEE), benzoyl-L-arginine ethyl ester (BAEE), benzoyl-L-arginine and L-lysine ethyl ester were a gift from Mr I. Jacobson. L-Tryptophan methyl ester, acetyl-L-tyrosine, L-tryptophan amide, acetyl-D-tryptophan amide were a gift from Dr S. Blumberg. L-Leucine ethyl ester, L- and D-methionine methyl ester, glycylglycine, L-alanyl-L-alanine, L- methionyl-L-methionine, disodium *p*-nitrophenyl phosphate, β-glycerophosphate, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) were products of Sigma Chemical Corp., St. Louis. L-amino acids were products of Mann Research Laboratories, N.Y. All reagents used were of analytical grade.

Lysosome enriched fraction

Method A. Male CR strain rats (100–300 g) were starved for 16–24 h and killed by skull fracture. Livers were excised and rinsed with ice-cold 0.45 M sucrose, 0.5 mM EDTA, pH 7.2. The livers were minced and homogenized in 2.5 vol. of the same solvent with a Teflon-on-glass Potter-Elvehjem-type homogenizer. The homogenate was further diluted with the sucrose solution to a final dilution of 8:1 (w/w), filtered through cheese cloth and subjected to differential centrifugation in a Sorvall RC2-B automatic refrigerated centrifuge. (All centrifugations in Methods A and B were carried out at 4 °C). The homogenate was centrifuged at $650 \times g$ for 10 min. The $650 \times g$ supernatant was centrifuged at $3300 \times g$ for 10 min. The $3300 \times g$ supernatant fraction was then centrifuged for 20 min at $16300 \times g$. The resulting pellet was resuspended in ice-cold 0.6 M sucrose, 0.5 mM in EDTA, pH 7.2, and centrifuged for 30 min at $5900 \times g$. The $5900 \times g$ supernatant fraction was centrifuged again at $16300 \times g$ for 20 min and the pellet obtained was resuspended in 0.6 M sucrose, pH 7.0. The specific activity of acid phosphatase in the lysosome enriched fraction assayed with β-glycerophosphate as substrate was higher than that of liver homogenate by a factor of 17–23.

Method B. Minced livers of non starved rats (details as above) were homogenized in 3 vol. of ice-cold 0.45 M sucrose, 0.5 mM EDTA, pH 7, in a Waring blender at top speed for 20 s. The homogenate was subjected to a differential centrifugation as described above.

In most of the experiments to be reported, lysosomes prepared by Method B, were used. At a later stage in the study it was found that a higher yield of a lysosome enriched fraction of a similar specific activity of acid phosphatase could be obtained upon modifying the fractionation procedure (Method A). Starvation of the rats was included in order to reduce glycogen deposits.

Enzyme assays

Acid phosphatase activity was determined by the method of Shibko and Tappel[6] with sodium β-glycerophosphate as substrate. Release of PO_4^{3-} was measured by the method of Fiske and SubbaRow[7]. With *p*-nitrophenyl phosphate as substrate for acid phosphatase, assays were performed according to Shibko and Tappel[6] or as described in the legends of the respective figures.

The hydrolysis of ATEE, BAEE and several other amino acid esters (see text) was followed potentiometrically. A radiometer pH-stat consisting of the SBR-2C/SB4-1/TTA-3 titration assembly and the PHM 26C pH-meter was used. The titrant was 0.05 M NaOH.

Analysis of enzyme-reaction products by high voltage electrophoresis

Electrophoresis was carried out in a model LT-36 electrophoresis tank. E.C. 123 coolant, and an HV-5000 power supply (Savant Instruments). Pyridine acetate buffer, pH 3.5, was prepared from glacial acetic acid–pyridine–water (10:1:89, v/v/v). 25-μl aliquots of reaction mixtures (see legends to Figs 4 and 5) were applied to Whatman No. 3 MM paper. After electrophoresis at a gradient of 50 V/cm for 40 min the paper was dried and dipped in a ninhydrin solution (0.5%, w/v ninhydrin in 75% aqueous acetone). Color was developed by heating in a well ventilated oven. The stain was fixed by dipping the paper in a $Cu(NO_3)_2$ solution (1% saturated $Cu(NO_3)_2$, 0.02% HNO_3 (conc.) in acetone: ethanol (2:1)).

Protein determination

Protein was determined by the method of Lowry *et al.*[8] with bovine plasma albumin as standard.

Turbidity measurements

The decrease in turbidity of a lysosomal suspension with time was followed in a Gilford recording spectrophotometer 2400-S at a wavelength of either 540 or 420 nm.

RESULTS

Effect of amino acid esters on the latency of acid phosphatase

A lysosome-rich fraction from rat liver exhibits latency with respect to its acid phosphatase activity for several h after its isolation. 10–20% of the total activity in the fraction (measured in the presence of 0.1% Triton X-100 or after subjecting the fraction to 10 cycles of freeze–thawing) is usually available, *i.e.* accessible to substrate in the lysosomal suspension. When L-amino acid methyl esters at a concentration of 1–2 mM are added to a lysosome-rich fraction suspended in 0.25 M sucrose, 10 mM in sodium HEPES (pH 7) rapid increase in the available acid phosphatase activity is observed. The increase in available acid phosphatase activity in a lysosome rich suspension could be monitored continuously by following the rate of *p*-nitrophenol release from *p*-nitrophenyl phosphate.

With the L-amino acid esters tested, after a short lag period (1–3 min), the available acid phosphatase activity increased rapidly. An almost constant rate was observed after a 10 min incubation period. The potency of six L-amino acid esters is compared in Table I to that of the respective D-stereoisomers. The addition of L-amino

TABLE I

EFFECT OF AMINO ACID ESTERS ON THE LATENCY OF ACID PHOSPHATASE AND ITS RELEASE INTO THE MEDIUM

A lysosome rich fraction (Method A) containing 160 μg protein, was suspended at 25 °C in 0.25 M sucrose, 10 mM sodium HEPES, pH 7, 0.01 M *p*-nitrophenylphosphate and the specified concentration of the added amino acid ester. The rate of *p*-nitrophenol release was followed at 420 nm in a Gilford recording spectrophotometer 2400 S. Each assay was referenced against an identical suspension but devoid of *p*-nitrophenyl phosphate. After a lag period (differing in length from compound to compound) the rates of *p*-nitrophenol release became quite constant. The rates recorded at a time interval of 5 min between 10 to 15 min of incubation are compared with the corresponding rates in the presence of 0.1% Triton X-100. Total activity in the control amounted to an increase in absorbance at 420 nm of 0.187 units per min. A lysosome rich fraction (400 μg protein) was suspended in 1.1 ml of 0.25 M sucrose, 0.01 M sodium HEPES, pH 7, and 2 mM of the specified ester, at 25 °C, for 15 min. The suspensions were then centrifuged for 30 min at 15000 $\times g$ and the resulting supernatants were assayed for acid phosphatase activity using 0.01 M *p*-nitrophenyl phosphate as substrate. The rates of *p*-nitrophenol release were followed at 420 nm in a Gilford spectrophotometer. Activities were compared to total activities obtained in the presence of 0.1% Triton X-100.

Amino acid ester added	*Concentration of added ester (mM)*	*% Rate at 10–15 min of total rate in 0.1% Triton X-100*	*Enzyme activity released in the medium (%)*
None		12	5
None*			53
L-Alanine methyl ester	2	54	36
D-Alanine methyl ester	2	13	6
L-Phenylalanine methyl ester	1	44	
L-Phenylalanine methyl ester	2	53	39
D-Phenylalanine methyl ester	2	16	7
L-Tyrosine methyl ester	2	44	28
D-Tyrosine methyl ester	2	16	6
L-Methionine methyl ester	0.4	75	
L-Methionine methyl ester	1	89	
L-Methionine methyl ester	2	110	70
D-Methionine methyl ester	2	13	8
Glycine methyl ester	2	28	
L-Leucine methyl ester	2	80	

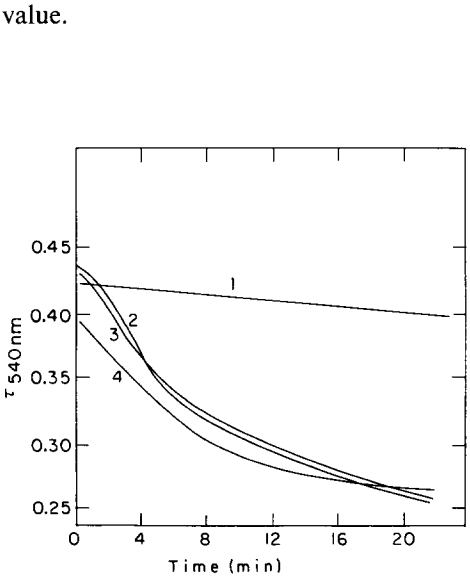
* Suspension exposed to 3 cycles of freezing and thawing.

acid esters to a lysosome-rich suspension leads to loss of latency of 44–100% after an incubation period of 10 min (shorter incubation periods were sufficient for the attainment of maximum effect with most compounds). In contrast the addition of D-amino acid esters does not lead to a significant increase in available enzymic activity. The fraction of acid phosphatase activity released into the medium, was estimated by subjecting the lysosomal suspension to a 30 min centrifugation at 15000 $\times g$. 60–70% of the available activity was found in the supernatant. Three cycles of freeze–thawing result in the release of 53% of lysosomal acid phosphatase into the medium (Table I).

The rate of rupture of lysosomes in the presence of L-amino acid esters was found to depend on the concentration of the ester in the concentration range tested (Table I).

Effect of amino acid esters on the turbidity of lysosome-rich fractions

The decrease in turbidity of lysosome suspensions in the presence of amino acid esters is illustrated in Fig. 1 for L-Ala-OMe, L-Leu-OMe and ATEE. Three distinct phases are common to all compounds tested. An initial lag period varying in length (see Figs 1 and 3), a rapid decrease in turbidity with a concomitant loss of latency and release of enzymes into the suspending medium, and a phase in which enzyme activation is complete and changes in turbidity of the remaining vesicles, ghosts or membrane fragments take place at a slow rate. The limiting values of the turbidity after 90 min of exposure of lysosomal suspensions to either of the above compounds are 30–40% of the corresponding initial values. Addition of Triton X-100 at a final concentration of 0.2% reduced the turbidity of a lysosome-rich suspension practically to zero. Ten cycles of freeze–thawing reduced it to approximately 25% of its initial value.



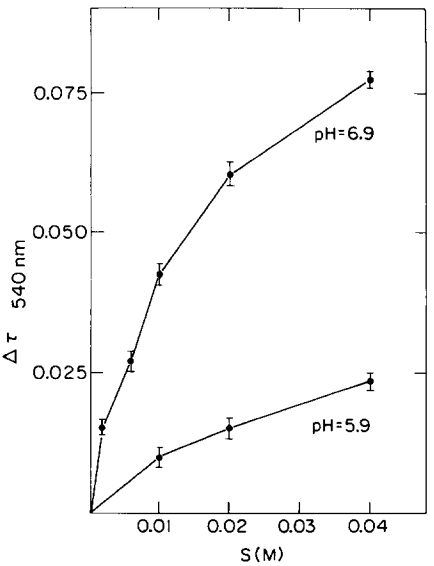


Fig. 1. Effect of L-amino acid esters and time on turbidity (τ) of lysosome-rich suspensions. 1, control; 2, L-Ala-OMe; 3, L-Leu-OMe; 4, ATEE. 75 μg of lysosomal protein (Method B) were suspended in 0.25 M sucrose, 0.05 M in sodium phosphate buffer, pH 7, and 0.02 M in the specified ester. Incubation was carried out at 25 °C.

Fig. 2. Effect of L-Ala-OMe concentration and medium pH on the rate of decrease of turbidity (τ) of lysosome-rich suspensions. 50 μl of lysosomes isolated by Method B (approx 75 μg protein) were suspended in 0.25 M sucrose, 0.05 M sodium phosphate buffer, and L-Ala-OMe at a specified concentration. The decrease in turbidity of the suspensions was traced with a Gilford spectrophotometer. Initial rates are expressed as $\Delta\tau_{540\ nm}$ per 4 min taken for the interval 1 to 5 min of the incubation at 25 °C. An initial τ of 0.275 and 0.32 was recorded for the same amount of lysosomal fraction at pH 6.9 and pH 5.9, respectively.

The ability of amino acid derivatives to lower the turbidity of a lysosomal suspension could be compared on the basis of the relative decrease in turbidity after incubation for fixed periods of time (see Table II). Absolute values of the effect were not reproducible from one lysosomal preparation to another, but the decrease in turbidity relative to the potent L-Phe-OMe could be used to compare data obtained from different preparations.

TABLE II

COMPOUNDS AFFECTING THE TURBIDITY OF A LYSOSOMAL SUSPENSION

50 μl of a lysosome rich fraction prepared by Method B (approx. 75 μg protein) were suspended in 1 ml of 0.25 M sucrose, 0.05 M in sodium phosphate buffer, pH 7, at 25 °C. The suspending medium was 0.02 M (Groups I and II) or 0.01 M (Group III) in the specified compound. The decrease in turbidity of the suspensions, at 540 nm, was traced simultaneously for one control and two test compounds. Groups I and II had the same control values. 100% turbidity decrease is the difference between the initial turbidity recorded for each lysosomal suspension and the value obtained upon incubation with L-Phe-OMe for 50 min.

Compound added	*Relative decrease in turbidity*	
	% after 10 min	*% after 20 min*
I		
None	5	10
Glycine methyl ester	18	40
L-Alanine methyl ester	47	64
D-Alanine methyl ester	10	17
L-Leucine methyl ester	50	65
L-Phenylalanine methyl ester	46	62
D-Phenylalanine methyl ester	10	25; 37
D-Tyrosine methyl ester	12	28
L-Lysine ethyl ester	Trace	
II		
Acetyl-L-tyrosine ethyl ester	48	58
Benzoyl-L-arginine ethyl ester	12	27
Acetyl-D-tryptophan amide	11	20
L-Tryptophan amide	6	25
III		
None	12	21
L-Alanine methyl ester	20	45
L-Tyrosine methyl ester	19	58
L-Tyrosine ethyl ester	44	95
L-Tryptophan methyl ester	70	91
L-Phenylalanine methyl ester	52	80

Effect of pH, ester concentration and ionic strength on the interaction of lysosomes with L-*amino acid esters*

(*a*) *pH and concentration of test compounds.* The dependence of the rate of decrease of the turbidity of a lysosomal suspension on the concentration of L-Ala-OMe at two different pH values of the suspending medium is given in Fig. 2. In the concentration range tested , the rate of decrease in turbidity was both concentration and pH dependent. In order to obtain the same initial rate of decrease in turbidity at pH 5.9 as at pH 6.9, 9–10-fold higher concentrations of L-Ala-OMe were needed. The decrease in turbidity of a lysosome suspension upon incubation with 5–40 mM of L-Ala-OMe at pH 8 exceeded by far the corresponding decrease observed at pH 6.9. The turbidity reached a final plateau value within a period of less than 5 min, thus initial rates of decrease in turbidity could not be compared with those obtained at pH 6.9 and 5.9.

(*b*) *Ionic strength dependence.* The dependence of the ability of three compounds,

L-Ala-OMe, L-Phe-OMe and L-Trp-OMe to affect lysosomal integrity on ionic strength at a constant osmolarity of the suspending medium is given in Fig. 3. It can readily be seen that as the concentration of NaCl in the medium increases (0–0.3 M), the susceptibility of lysosomes to the test compound increases. The effect is less pronounced with the alanine ester and is dramatic for the tryptophan ester. Both sucrose at 0.6 M and NaCl at 0.3 M give good osmotic protection to lysosomes[5,9].

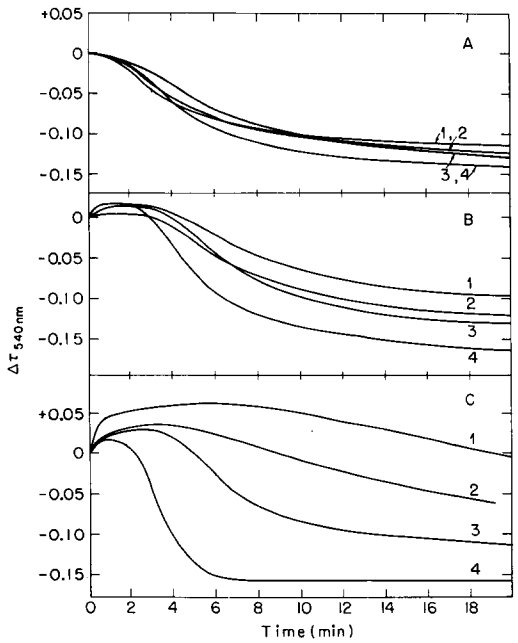


Fig. 3. Effect of ionic strength, at constant osmolarity, on the rate of decrease of turbidity of a lysosome rich suspension. A, 0.02 M L-Ala-OMe; B, 0.02 M L-Tyr-OMe; C, 0.02 M L-Trp-OMe. Four suspending media were used: (1) 0.6 M sucrose; (2) 0.45 M sucrose + 0.075 M NaCl; (3) 0.3 M sucrose + 0.15 M NaCl, and (4) 0.3 M NaCl. All solutions were also 0.02 M in sodium phosphate buffer, pH 7. The initial turbidity of the suspension was 0.280 absorbance units at 540 nm. In each run at a specific osmolarity of the suspending medium, the effect of the three compounds referenced against the control of lysosomes in the absence of the compound, was followed. The temperature was kept constant at 25 °C.

Amino acid esters as substrates of lysosomal enzymes

(*a*) *Ester hydrolysis measured potentiometrically.* The rates of hydrolysis of several of the amino acid esters tested were measured potentiometrically. A three times freeze–thawed lysosomal preparation (approx. 100 μg protein) incubated with the specified compounds (at a concentration of 0.02 M, 0.5 M in NaCl, pH 7, and at a temperature of 25 °C) gave the following rates of OH^- consumption: ATEE, 0.625 μmole/min; BAEE, 0.33 μmole/min; L-Leu-OMe and L-Phe-OMe, approximately 0.07 μmole/min; L-Trp-OMe, about 0.03 μmole/min; L-Tyr-OMe, 0.025 μmole/min; L-Ala-OMe, D-Phe-OMe, D-Tyr-OMe, a maximum rate of 0.01 μmole/min. Control rates of non enzymatic hydrolysis were substracted from the reaction rates.

The pH activity profiles of BAEE and ATEE hydrolysis by a lysosomal preparation were similar, showing an optimum activity at pH 7.5. The ratios of the rates of

hydrolysis at pH 7 and pH 6 were similar for BAEE, ATEE, L-Leu-OMe and L-Phe-OMe (approx. 1.8–1.9); thus all of these compounds are hydrolyzed by an enzyme that has an optimum activity in the neutral pH range. (At higher pH values there is a rapid enzyme inactivation.)

(*b*) *An analysis of the enzyme reaction products.* The pattern of lysosomal enzyme activity with L- and D-amino acid esters as substrates is given in Figs 4 and 5. Both L- and D-phenylalanine methyl esters are totally degraded to the respective amino acids. However, the enzymic reaction of L-alanine methyl ester results in the appearance of

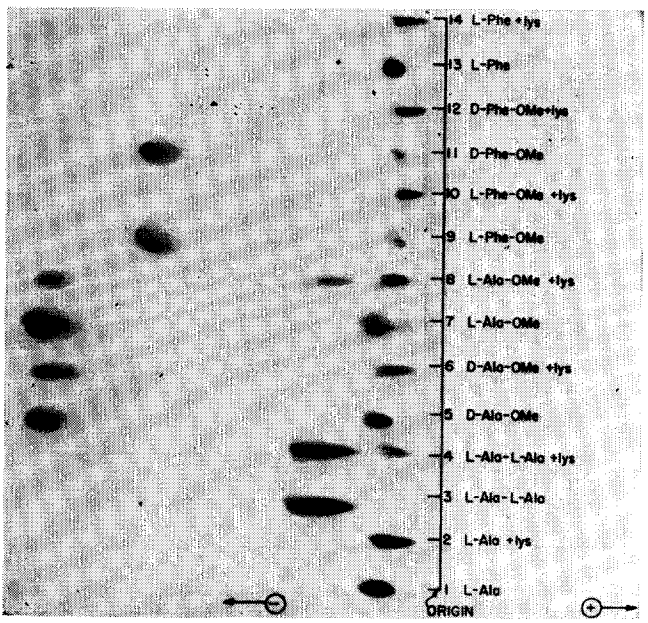


Fig. 4. Analysis of enzyme reaction products. The specified compounds at a concentration of 0.02 M (0.02 M in sodium phosphate buffer, pH 7.0) were incubated with or without a 3 times freeze–thawed lysosome-rich fraction isolated according to Method A (0.6 mg protein per ml) for 3.5 h at 37 °C. Aliquots of 25 μl were then spotted on a 3 MM Whatman paper and subjected to high voltage electrophoresis (see Methods). lys, denoted incubation with the lysosomal fraction.

a new species, presumably alanylalanine (Fig. 4). The dipeptide was shown to be susceptible to enzyme hydrolysis by the lysosomal preparation (Fig. 4), its breakdown is apparently slower than its generation under the assay conditions D-Ala-OMe if degraded at all, did not yield a dipeptide. A shorter period of incubation (1 h) gave essentially the same result with respect to the degradation pattern of the 4 compounds. When the incubation was carried out at pH 5.4 (0.02 M of sodium acetate) instead of pH 7, similar results were obtained. However, the rates of the enzymic reactions were lower at pH 5.4 than at pH 7. Incubation of L- and D-tyrosine methyl esters with a lysosomal fraction under the same experimental conditions results in the total hydrolysis of the ester to the free amino acid. By reducing the incubation period with D- and L-Tyr-OMe and D- and L-Phe-OMe, it was found that the L-stereoisomers were hydrolyzed at a higher rate than the D-stereoisomers.

In order to clarify whether dipeptide formation was a common intermediate

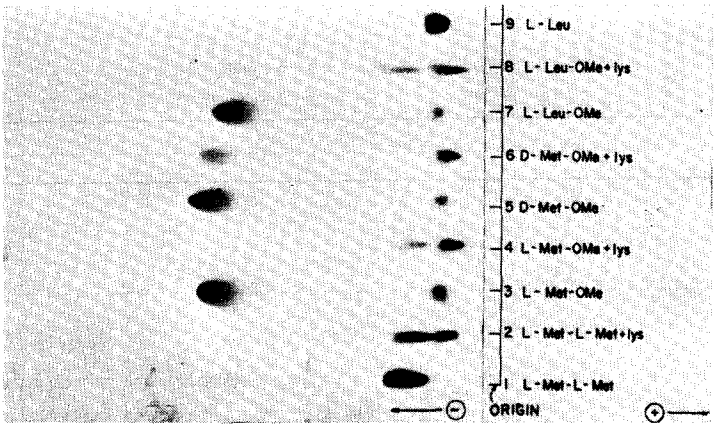


Fig. 5. Analysis of enzyme reaction products. The details of the experiments are those described in the legend to Fig. 4. Incubation time 1.5 h.

step in the hydrolysis of aliphatic amino acid esters, three additional L and D esters were tested (Fig. 5). After an incubation period of 90 min all of the L-methionine methyl ester was converted to either the dipeptide or the free amino acid. A significant amount of the dipeptide was found in the incubation mixture even though dimethionine is hydrolyzed at a significant rate by the lysosomal preparation (see Fig. 5). D-Met-OMe was not degraded significantly. L-Leu-OMe was also almost totally degraded with a concomitant appearance of a new species —presumably the dipeptide. Gly-OMe was degraded to a lesser extent, leading to the appearance of a dipeptide—glycylglycine and another spot of unknow composition (not shown).

DISCUSSION

The data presented in Tables I and II show that esters of amino acids are potent in bringing about the rupture of lysosomes. The capacity of amino acid esters to release acid phosphatase into the medium and decrease the turbidity of a lysosomal suspension was found to be stereo-specific, *i,e*, only esters of L-amino acids were effective in damaging lysosomal integrity. The correlation between availability of enzyme activity and decrease in turbidity of a lysosomal suspension (Tables I and II) makes possible the choice of the latter criterion as a relatively convenient measure of the state of lysosomes, since it obviates the need for correcting for effects of enzyme substrate on lysosomal stability and for effects of test compounds on enzyme activity.

Because these experiments were carried out in media of high osmolarity (0.25 or 0.6 M in sucrose) and a relatively low concentration of the added esters the possibility that rupture of lysosomes was caused by the small change in the chemical potential of the intralysosomal water resulting from simple ester permeation, can be excluded. Two alternate explanations are presented:

Lysosomal rupture due to a specific interaction of the solute with a structural membrane component

Solute adsorption onto membrane sites or penetration into the lipid bilayer regions may lead to pore formation, to increase in membrane surface area, or to

increase in the degree of micelle formation of the lipids within the membrane[10] and thus bring about changes in the permeability properties of the membrane to low molecular weight compounds. If for example the lysosomal membrane becomes permeable to sucrose, sucrose penetration will drive an inward flow of water, the lysosome will swell and eventually burst releasing its intralysosomal constituents to the medium.

The dependence of the decrease in turbidity of a lysosomal suspension on the concentration of L-Ala-OMe at two different pH values supports the suggestion that at least the primary step in the process involves membrane penetration by an unionized molecular species. The pK value of the NH_2-group of L-Ala-OMe is 7.76 (ref. 11). The concentration of uncharged species in therefore highly pH-dependent in the pH range tested, *i.e.* 12.1 and 1.4% of the compound are in the unionized form at pH 6.9 and 5.9, respectively. A factor of 9 in the concentration of the unionized species is in accord with the displacement observed in the concentration dependence of the rate of decrease in turbidity at the two pH values (Fig. 2). An unionized molecule has a higher probability of penetrating biological membranes by passive diffusion than a charged molecule. The fact that L-lysine ethyl ester and BAEE do not affect lysosomal integrity may be attributed to the fact that these esters are almost completely charged at the neutral pH range.

NaCl has been shown to have less osmotic protecting capacity for lysosomes than sucrose[5,9]. If membrane permeability towards sucrose and NaCl is changed to a different extent by the interaction with various compounds, then one could expect variations in the extent of the effect of NaCl concentration on lysosomal lysis, upon incubation with different compounds, in line with observations given in Fig. 3.

Some indication that interaction of α-amino acid esters with membrane phospholipids may lead to changes in the permeability properties of the membrane is obtained from studies on model systems (Goldman, R., unpublished results). It has been found that lecithin vesicles become more permeable to glucose upon addition of 0.02 M of several of the potent esters used in this study, *i.e.* ATEE, L-Tyr-OMe, L-Leu-OMe and L-Trp-OMe. Changes in the initial rate of glucose diffusion of up to 2-fold increase in the rate were observed. However, L-Ala-OMe, a very potent lysosomal labilizer, failed to affect the bilayer properties. Furthermore, the stereospecificity of the ester effect on lysosomal membranes requires that postulated solute–membrane interactions must be mediated by ester binding to membrane protein.

Lysosomal rupture resulting from an intralysosomal enzyme reaction on compound

A second interpretation of the experimental data is based on the following considerations. Assume that a compound penetrates into lysosomes and is degraded *in situ* by the enzyme composite of the particle. As a result of the intralysosomal enzymic hydrolysis, a gradient in concentration of the compound–substrate will be preserved across the membrane, *i.e.* a passive diffusion of substrate will continually provide substrate to the interior of the lysosome. If, on the other hand, the product of the reaction is not permeable or is much less permeable than the substrate, it will accumulate within the particle leading to significant differences in osmotic pressure across the membrane. Osmotic pressure differences lead to an inward flow of water, swelling and an eventual rupture of the membrane-limited particle.

All of the potent esters have been shown to be hydrolyzed by a freeze–thawed lysosomal fraction. The data presented regarding the products of lysosomal enzyme

reaction on the tested esters suggests that at least two different enzyme species are involved. The aliphatic L-amino acid esters were shown to undergo a step of transpeptidation yielding the corresponding dipeptides, which were further hydrolyzed to free amino acids. The aliphatic D-amino acid esters did not undergo a transpeptidation reaction and if any hydrolysis to the corresponding amino acids took place, it could not be detected under the experimental conditions used. The aromatic L-amino acid esters as well as the aromatic D-amino acid esters were both hydrolyzed to the corresponding amino acids, the rate of hydrolysis of the L-stereoisomer exceeding that of the D-stereoisomer.

The correlation between enzyme reaction on the various esters and their potency in damaging lysosomal integrity is strongly suggestive that the mechanism of rupture has to do with a primary enzyme reaction in the intact granules. The L-aliphatic and aromatic amino acid esters were found to be very potent in rupturing lysosomal populations, whereas the D analogues of the aliphatic amino acid esters did not possess this activity (Tables I and II). The fact that the D-aromatic amino acid esters brought about a certain decrease in lysosomal turbidity after an incubation period of 20 min (Table II) might be indicative of the slow enzyme hydrolysis that these compounds undergo. In this connection it is worth noting that whereas L-Trp-OMe is a very potent lysosomal labilizer, the amide analogue, L-Trp-NH_2, is remarkably less effective (Table II).

L-Ala-OMe was found to be more potent in rupturing lysosomes when incubated with a lysosome rich fraction at pH 7 than at pH 6. This may reflect, besides a higher concentration of a permeating species (as discussed before), a higher rate of intralysosomal ester degradation at the higher extralysosomal pH.

The effect of medium ionic strength on the rate of lysosomal rupture (Fig. 3) may be attributed to a different dependence of intralysosomal enzyme activity on ionic strength. NaCl permeates intact lysosomes[9,12], and a strong NaCl activation of ATEE hydrolysis by freeze–thawed lysosomal preparations was observed.

The assumption that products of intralysosomal hydrolysis do not permeate lysosomal membranes readily is supported by the finding that free amino acids provide good osmotic protection to a lysosomal suspension[5]. We have estimated that lysosomes have the capacity to generate 0.05 μmole of amino acid products of esterase activity per min in 0.1 μl of lysosomal osmotic space. This generation of 0.5 M product per minute would be more than sufficient to bring about high internal osmotic pressures and eventually rupture of lysosomes. Direct measurements of the concentration and location of esters and their reaction products within the lysosome will be useful for the further evaluation of these and other explanations for ester mediated lysosomal destruction.

An intralysosomal hydrolysis of amino acid esters can effect lysosomal integrity by yet another mechanism. H^+ are liberated in the course of ester hydrolysis. As a result, the internal pH of the lysosome may drop[13–15] leading to its rupture. Lysosomes rupture readily when suspended in a medium of a pH lower than 6[3].

It is noteworthy that since lysosomes exhibit a certain concentrative capacity towards the effective compounds[12,16] then substrates will be available at the catalytic site in a concentration exceeding that predicted by the balance of an inward diffusional flow and hydrolysis.

Morphological observations on dividing and non-dividing cells are consistent with

the view that release of lysosomal material into the cytoplasm acts as a trigger initiating division in a cell that is ready for it (reviewed by Allison[17]). Furthermore, an abnormal release of acid hydrolases from lysosomes has been implicated in the pathology of a variety of disorders (review articles[17,18]). The primary cause for release of lysosomal enzymes *in vivo* is still an enigma in most of the cases. The possibility that low molecular weight metabolites effect lysosomal integrity seems worth looking into.

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