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Intralysosomal hydrolysis of glycyl-L-phenylalanine 2-naphthylamide

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1. Glycyl-L-phenylalanine 2-naphthylamide (Gly-L-Phe-2-NNap), a cathepsin C substrate, induces an increase of the free and unsedimentable activities of this enzyme when incubated with a total mitochondrial fraction of rat liver. 2. 1 mM-$ZnSO_4$ considerably inhibits the cathepsin C total activity, measured with Gly-L-Phe-2-NNap as the substrate, in the presence of Triton X-100. The inhibition is markedly less pronounced when the free activity is determined; a high activity remains that depends on the integrity of the lysosomes; it decreases as the free activity of *N*-acetylglucosaminidase increases when lysosomes are subjected to treatments able to disrupt their membrane. 3. Cathepsin C activity is reduced when thioethylamine hydrochloride is omitted from the incubation medium. Under these conditions at 37°C, the free activity equals the total activity, although the lysosomes are intact, as indicated by the low free activity of *N*-acetylglucosaminidase. 1 mM-$ZnSO_4$ strikingly inhibits the total activity, whereas more than 80% of the free activity remains. 4. These observations are presented as evidence that Gly-L-Phe-2-NNap can possibly cause a disruption of the lysosomes as a result of its hydrolysis inside these organelles. In the presence of $ZnSO_4$, intralysosomal hydrolysis becomes apparent, owing to a preferential inhibition by Zn^{2+} of extralysosomal hydrolysis; in the absence of thioethylamine hydrochloride, it is measurable because the disruption of lysosomes by Gly-L-Phe-2-NNap is delayed as a result of a slow-down of the reaction. 5. The usefulness of Gly-L-Phe-2-NNap and related dipeptidyl naphthylamides in lysosomal-membrane-permeability studies is emphasized.

As has been shown by Goldman & Kaplan (1973) and Goldman & Naider (1974), methyl esters of certain amino acids and dipeptides induce a loss of the latency of rat liver lysosomal acid phosphatase. Those authors have proposed that this phenomenon originates from a penetration of these substances into lysosomes, where they are hydrolysed by an esterase into compounds that do not easily diffuse through the lysosomal membrane. This hypothesis has been substantiated by the observations of Reeves (1979), who showed that the incubation of lysosomes in the presence of leucine methyl ester leads to an accumulation of leucine within the organelles.

Such molecules able to penetrate into, and be hydrolysed within, the lysosomes can be profitably used to study intralysosomal hydrolysis and the permeability of the lysosomal membrane. In looking for other substances whose intralysosomal hydrolysis could possibly be measured, our attention was aroused by dipeptidyl naphthylamides, which are used as substrates for cathepsin C (McDonald *et al.*, 1969). In the work presented here we show that Gly-L-Phe-2-NNap can cause a loss of lysosome integrity and that it is possible to show the intralysosomal hydrolysis of such a compound.

A preliminary report of this work was presented at the 74th Meeting of the American Society of Biological Chemists, San Francisco, 5–9 June 1983.

Abbreviations used: abbreviations for peptide derivatives are according to the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1971). 2-Naphthylamide substitution at the carboxy group is indicated by -2-NNap.

Experimental

Tissue fractionation

Experiments were performed with male Wistar rats weighing 200–300g. The animals were decapitated after food deprivation for 20h. The livers were immediately removed, chilled in ice-cold 0.25M-sucrose, and a mitochondrial fraction, corresponding to the sum of the 'M' and 'L' fractions of de Duve *et al.* (1955) was prepared.

Enzyme assays

For assay of cathepsin C (EC 3.4.14.1) the enzyme preparation was generally incubated at 37°C, in a total volume of 2ml containing the dipeptidyl naphthylamide at the required concentration, 0.05M-acetate or -Tris buffer, 5mM-thioethylamine hydrochloride, 0.25M-sucrose and, for total-activity determination, 0.05% Triton X-100. The incubation time never exceeded 10min. The reaction was stopped by addition of 1ml of 10% (w/v) trichloroacetic acid. The released naphthylamine was determined by colorimetry as described by Barrett (1972). *N*-Acetylglucosaminidase (EC 3.2.1.30) was measured by the method of Vaes (1966).

Results

Effect of Gly-L-Phe-2-NNap on cathepsin C latency

The free activity of cathepsin C measured with Gly-L-Phe-2-NNap as the substrate depends strikingly on the pH and on the concentration of the dipeptidyl naphthylamide, as illustrated in Fig. 1. The cathepsin C latency is not affected by substrate concentration at pH5; it vanishes progressively with the rise of the substrate concentration at higher pH; the higher the pH the more marked is the substrate effect. The loss of latency caused by Gly-L-Phe-2-NNap results from a true release of the enzyme into the medium; the non-sedimentable activity of cathepsin C increases like the free activity when the Gly-L-Phe-2-NNap concentration and the pH increase. Gly-L-Phe-2-NNap causes a loss of the latency not only of cathepsin C but also of other lysosomal enzymes, for example *N*-acetylglucosaminidase and β-galactosidase. Dipeptidyl naphthylamides that are not substrates for cathepsin C (Gly-L-Pro-2-NNap and Gly-D-Phe-2-NNap), or that contain a charged residue at pH 6 (Gly-L-Arg-2-NNap and L-Asp-L-Ala-2-NNap), do not exhibit any effect on cathepsin C latency.

Intralysosomal hydrolysis

The experimental demonstration of intralysosomal hydrolysis of a substrate added to the medium raises a problem. Indeed, lysosome preparations

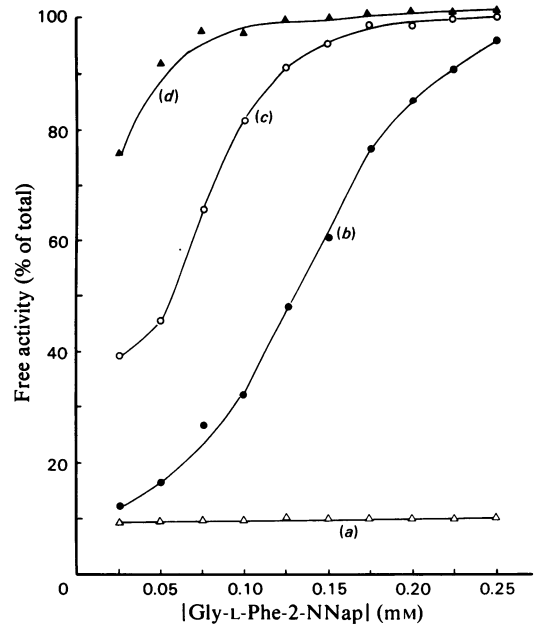

Fig. 1. *Effect of substrate concentration and of pH on cathepsin C free activity*

A mitochondrial fraction was incubated for 10min at 37°C in the presence of various concentrations of Gly-L-Phe-2-NNap, 0.05M-acetate (pH5 and pH6) or -Tris buffer (pH6.5 and 7), 0.25M-sucrose, 5mM-thioethylamine hydrochloride and, for total activity measurements, 0.05% Triton X-100. The activities are given as percentages of the total activity. (*a*) pH5; (*b*) pH6; (*c*) pH6.5; (*d*) pH7.

contain a significant proportion of damaged organelles having released their enzymes. Moreover, when a deterioration of lysosomes takes place in the presence of the substrate, more and more activity becomes free with time. Therefore, under these conditions, it is difficult to distinguish between an activity occurring inside the granules and one resulting from the released enzyme. We have tried to solve this problem in the following two ways. (1) By adding to the incubation medium a strong inhibitor of cathepsin C that could not diffuse quickly into the lysosomes. We reasoned that, in the presence of such a substance, most of the cathepsin C outside the lysosomes would be inhibited when that present inside the granules would be less inhibited or uninhibited. Since the lysosomal membrane is poorly permeable to salts (Reijngoud & Tager, 1977), we selected $ZnSO_4$ as the inhibitor (Misaka & Tappel, 1971). (2) By measuring the hydrolysis of Gly-L-Phe-2-NNap by intact lysosomes, in the absence of thioethylamine hydrochloride. Since the activity of cathepsin C is

markedly reduced in these conditions, it would be expected that the disruption of the lysosomes could be delayed if, as it is supposed, this depends mainly on the hydrolysis of Gly-L-Phe-2-NNap inside the organelles. Moreover, the intra- and extra-lysosomal hydrolysis may be inequally influenced by thioethylamine, at least at the start of the reaction, if the lysosomal membrane does not allow a rapid penetration of this molecule into the lysosomes.

Cathepsin C activity in presence of 1 mM-$ZnSO_4$

$ZnSO_4$ at 1 mM inhibits quasi-totally the cathepsin C present in rat liver mitochondrial fractions measured in the presence of Triton X-100 (total activity). Contrarily, a very significant activity is apparent when free cathepsin C is determined (Fig. 2). The reaction rate remains quasi-constant up to 1–1.5 min, then decreases. $ZnCl_2$ and zinc acetate have the same effect as $ZnSO_4$. Results are identical if, instead of a total mitochondrial fraction, highly purified lysosomes (Wattiaux *et al.*, 1978) are incubated with Gly-L-Phe-2-NNap and 1 mM-$ZnSO_4$.

That such an activity is linked to the integrity of the lysosomes is illustrated in Fig. 3. In these experiments, lysosomes have been subjected to successive freezing and thawing, to hypo-osmotic treatments or mechanical disruption in an Ultra-Turrax homogenizer. The extent to which the lysosomes deteriorated was assessed by measuring the free activity of *N*-acetylglucosaminidase. Obviously the free activity of cathepsin C, determined in the presence of 1 mM-$ZnSO_4$, depends on the latency of *N*-acetylglucosaminidase; it decreases as the free activity of this enzyme increases. In another experiment the free activity of *N*-acetylglucosaminidase was determined together with that of cathepsin C with 1 mM-$ZnSO_4$, at increasing times after lysosome addition. During the initial period, when hydrolysis of Gly-L-Phe-2-NNap is linear, the free activity of *N*-acetylglucosaminidase is quasi-constant and corresponds to the basal free activity ($\approx$10% of the total). Later it progressively increases and, in parallel, the rate of Gly-L-Phe-2-NNap hydrolysis decreases.

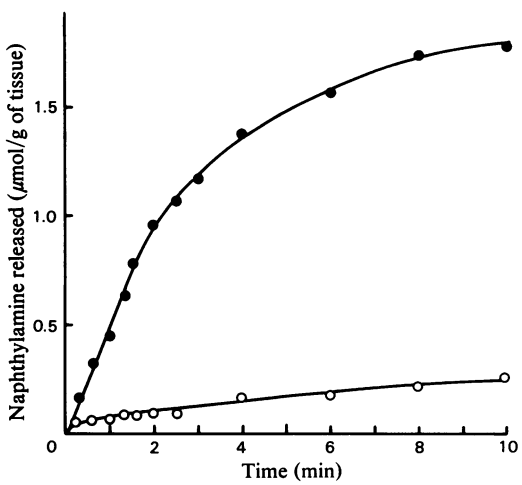

Fig. 2. *Effect of Zn^{2+} on Gly-L-Phe-2-NNap hydrolysis*
The Figure shows the hydrolysis of Gly-L-Phe-2-NNap by a mitochondrial fraction incubated for increasing times at 37°C in presence of 0.25 mM-Gly-L-Phe-2-NNap, 0.05 M-acetate buffer, pH 6, 0.25 M-sucrose, 5 mM-thioethylamine hydrochloride, 1 mM-$ZnSO_4$ and, for total-activity measurements, 0.05 Triton X-100. ●, Activity in the absence of Triton X-100 (free activity); ○, activity with Triton X-100 (total activity).

It should be noted that such a difference in the inhibition by Zn^{2+} of cathepsin C, according to whether the free or the total activities are measured, is not observed at pH 5 or at any pH when Gly-L-Arg-2-NNap is used as the substrate. In this case, 1 mM-$ZnSO_4$ exhibits the same inhibition of the free and the total activities.

Cathepsin C activity in the absence of thioethylamine hydrochloride

For full activity, purified cathepsin C requires the presence of a thiol and of Cl^-. We used a method where both requirements are satisfied by adding thioethylamine hydrochloride in the incubation medium (Barrett, 1972). In the absence of this compound, cathepsin C activity is about 10 times lower. (In fact we have more recently found that, with a crude granule fraction, activation is essentially due to Cl^- and not to thiol.) Fig. 4 allows one to compare the evolution with time of the free and the total cathepsin C activities, in the absence of thioethylamine hydrochloride, at different temperatures. At 37°C, the free and the total cathepsin C activities are equal; when the temperature decreases, the free activity becomes progressively less than the total activity. In these experiments, *N*-acetylglucosaminidase free activity was measured to monitor the integrity of lysosomes; it did not exceed 10% of the total activity whatever the temperature might be, indicating that lysosomes did not release their enzymes into the medium. Thus free and total activities of cathepsin C are distinctly dependent on temperature. If the logarithms of the reaction rates are plotted against the reciprocals of the absolute temperatures, straight lines are obtained for both the free and the total activities; calculation of the activation energy gives 75.3 kJ (18 kcal)/mol for the free activity and 31.4 kJ (7.5 kcal)/mol for the total activity.

Free and total activities without thioethylamine hydrochloride, although being equal at 37°C, are differently susceptible to Zn^{2+}. As shown in Fig. 5,

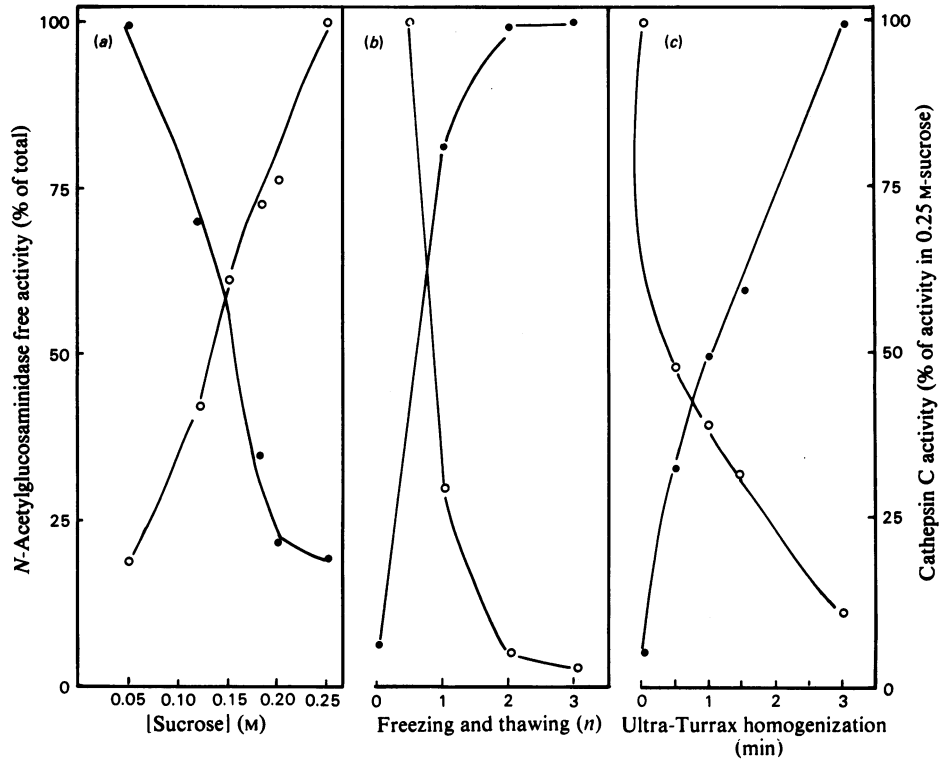

Fig. 3. *Lysosome integrity and cathepsin C activity measured in the presence of 1 mM-$ZnSO_4$*
A mitochondrial fraction was subjected to different treatments causing disruption on the lysosomal membrane. Cathepsin C and *N*-acetylglucosaminidase were assayed in the different preparations in the presence of 0.25 mM-Gly-L-Phe-2-NNap, 0.05 M-acetate buffer, pH 6, 5 mM-thioethylamine hydrochloride, 8 mM-*p*-nitrophenyl *N*-acetylglucosaminide and 1 mM-$ZnSO_4$. (*a*) Hypo-osmotic rupture; the mitochondrial fraction was exposed to the sucrose concentrations indicated on the abscissa for 10 min at 0°C. After this treatment, the sucrose concentration was readjusted to 0.25 M. (*b*) Freezing and thawing: the mitochondrial fraction was frozen in liquid N_2 and thawed at 0°C one, two or three times (*n*); (*c*) mechanical disruption: the mitochondrial fraction was treated in an Ultra-Turrax homogenizer for the time indicated on the abscissa. ●, *N*-Acetylglucosaminidase free activity expressed as percentage of the activity obtained after maximal disruption of lysosomes; ○, cathepsin C activity expressed as percentage of the activity found with untreated granules.

1 mM-$ZnSO_4$ considerably inhibits the total activity, whereas more than 80% of the free activity remains.

Discussion

Our results show that Gly-L-Phe-2-NNap is able to suppress the latency of cathepsin C under certain conditions of pH and concentration. The fact that the loss of latency occurs concomitantly with a release of the enzyme in the medium indicates that a true disruption of the lysosomal membrane takes place. Our observations are in parallel with those made by Goldman & Kaplan (1973) and Goldman & Naider (1974) with methyl esters of amino acids and di- and tri-peptides, compounds to which dipeptidyl naphthylamides are related. Like these substances, Gly-L-Phe-2-NNap is a weak base (pK_a of the amino group = 7.5) and relatively hydrophobic; moreover, the bound amide can be rapidly hydrolysed by cathepsin C.

Evidence that such a hydrolysis can occur *in vitro* within the lysosomes is provided by the observations on cathepsin C activity exhibited in the presence of Zn^{2+} or in the absence of thioethylamine hydrochloride. In the presence of 1 mM-$ZnSO_4$ the free activity of cathepsin C, considerably higher than the total activity, indicates that the enzyme is to some extent protected against inhibition by Zn^{2+} at the start of the incubation. The relationship between this activity and the latency of *N*-acetylglucosaminidase (an indicator of lysosome integrity), strongly suggests that such protection is provided by the lysosomal membrane, and, therefore, that the cathepsin C free activity measured in the presence of Zn^{2+} results from the intralysosomal hydrolysis of the dipeptidyl naphthylamide. The fact that this effect of Zn^{2+} is not observed at pH 5, where the amount of

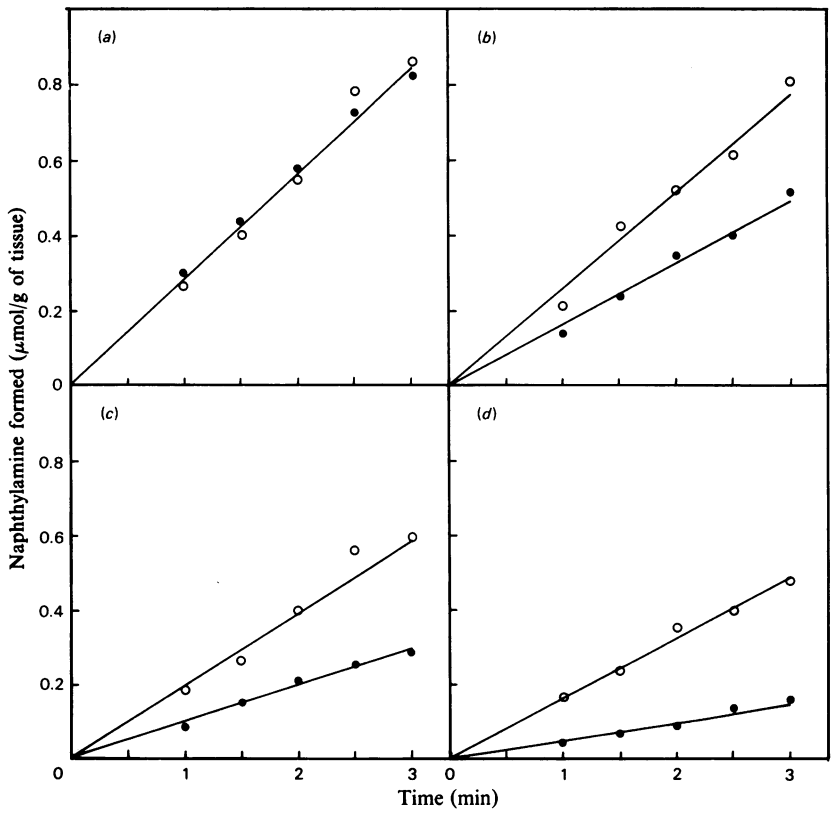

Fig. 4. *Influence of temperature on the free and total activities of cathepsin C in the absence of thioethylamine hydrochloride*
The mitochondrial fraction was incubated at different temperatures in the presence of 0.25mM-Gly-L-Phe-2-NNap, 8mM-*p*-nitrophenyl *N*-acetylglucosaminide, 0.05M-acetate buffer, pH6, 0.25M-sucrose and, for the total-activity measurements, 0.05% Triton X-100. (*a*) 37°C; (*b*) 32°C; (*c*) 27°C; (*d*) 22°C. ○, Total activity; ●, free activity. In these assays, the free *N*-acetylglucosaminidase activity never exceeded 10% of the total activity.

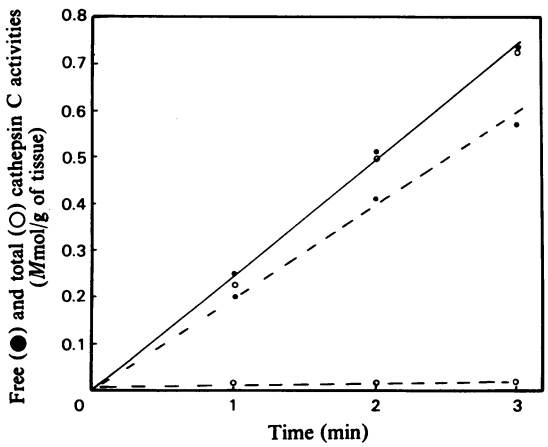

Fig. 5. *Effect of 1mM-$ZnSO_4$ on free and total cathepsin C activities in the absence of thioethylamine hydrochloride*
A mitochondrial fraction was incubated at 37°C in presence of 0.25mM-Gly-L-Phe-2-NNap, 0.05M-acetate buffer, pH6, and 0.25M-sucrose in the absence (——) or in the presence (----) of 1mM-$ZnSO_4$. For total activity, 0.05% Triton X-100 was added.

unprotonated Gly-L-Phe-2-NNap is markedly decreased, supports this interpretation. In the absence of thioethylamine at 37°C, cathepsin C free activity is equal to the total activity as soon as the reaction starts; *N*-acetylglucosaminidase free activity is low for several minutes, showing that most of the lysosomes remain intact. Therefore the high free activity of cathepsin C cannot originate from a release of the enzyme from the lysosomes and must be the result of intralysosomal hydrolysis. Additional evidence is provided by the differential effect of Zn^{2+} on the free and the total activities under these conditions.

As ascertained by the rate of Gly-L-Phe-2-NNap hydrolysis in the absence of thioethylamine, the diffusion rate of this compound through the lysosome membrane must be high at 37°C. Indeed, since free and total activities are equal, the substrate concentration within the lysosomes has to be the same as that in the outside medium, because the concentration used in our assay is in the region of the enzyme's K_m value. At lower temperatures the cathepsin C free activity be-

comes progressively less than the total activity; this suggests that the diffusion rate of unprotonated Gly-L-Phe-2-NNap is highly dependent on temperature. These results differ from those of Reeves (1979), which indicated that accumulation of leucine in lysosomes is apparently independent of the temperature over several minutes. On the other hand, they are in agreement with the observations of Stein (1967) and McElhaney *et al.* (1973), who showed a strong temperature-dependence of non-electrolyte diffusion through artificial and natural membranes.

The measurement of the free activity of cathepsin C with Gly-L-Phe-2-NNap in the absence of thioethylamine hydrochloride is a simple experimental method to study factors influencing the permeability of the lysosomal membrane when a permeation mechanism (de Duve *et al.*, 1974) is involved. Indeed, the intralysosomal hydrolysis of Gly-L-Phe-2-NNap (and possibly other dipeptidyl naphthylamides) is directly linked to the diffusion rate of the compound into the lysosomes. In our opinion such a method presents several advantages with respect to the one founded on the measurement of the accumulation of leucine inside the lysosomes after hydrolysis by esterase of its methyl ester (Reeves, 1979; Reeves & Reames, 1981). Firstly, it is surely easier to apply when multiple determinations have to be made. Its sensitivity could even be improved by the fluorimetric detection of naphthylamine, which, in addition, permits a continuous recording of the reaction. Secondly, the determinations are not affected by the efflux of hydrolysis products through the lysosomal membrane, as shown for leucine in Reeves's (1979) method. This simplifies the kinetic study of the process. Thirdly, cathepsin C is only located in lysosomes; esterase activity has a wider distribution in the liver. Amino acid methyl esters may be subjected to hydrolysis by esterases other than the lysosomal esterase when relatively crude granule preparations are used with a possible accumulation of amino acid into non-lysosomal organelles, e.g. vesicles of the endoplasmic reticulum that are endowed with a high esterase activity (Amar-Costesec *et al.*, 1974).

The determination of activity in the presence of Zn^{2+} is perhaps more restricted, owing to the possible effect of that ion on the lysosomal membrane (Ludwig & Chvapil, 1980; Pfeiffer & Cho, 1980).

There is another practical application for results presented here. Gly-L-Phe-2-NNap may be considered as a substrate able to cause the loss of lysosome integrity specifically. Indeed, its action absolutely requires an intralysosomal reaction that cannot occur in other organelles. This can be profitably used in investigations of subcellular localization, particularly for endocytosed molecules. Several workers have shown that, after endocytosis, substances can be distributed among non-lysosomal and lysosomal structures (for a review, see Steinman, 1983). All these structures may be endowed with relatively similar properties with respect to size, density and susceptibility to damaging agents. This may make it difficult to establish the distribution of endocytosed components among them by usual methods such as centrifugation and activation experiments. Looking for the possible release of an endocytosed molecule by Gly-L-Phe-2-NNap together with that of cathepsin C could be a choice method to distinguish between a lysosomal and a non-lysosomal location for this molecule.

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