The Inhibition of Macrophage Protein Turnover by a Selective Inhibitor of Thiol Proteinases

Elliott SHAW* and Roger T. DEAN†

Division of Cell Pathology, Clinical Research Centre, Watford Road, Harrow, Middx. HA1 3UJ, U.K.

(Received 13 June 1979)

1. A new inhibitor of thiol proteinases, benzyloxycarbonylphenylalanylalanine diazomethyl ketone (benzyloxycarbonylphenylalanyldiazomethane, Z-Phe-Ala-CHN₂) was added to cultured mouse peritoneal macrophages prelabelled with [¹⁴C]leucine. The degradation of protein was studied under conditions of basal proteolysis in the presence of 10% pig serum. After a lag of about 6 h a time- and dose-dependent inhibition of protein degradation was observed, up to a maximum of about 40%. 2. The inhibitor entered the cells with kinetics consistent with entry by pinocytosis, giving access to the lysosomal system. 3. Intracellular cathepsin B was almost completely inactivated after 90 min of exposure of the culture to 0.1 mM-inhibitor. 4. The inhibition of proteolysis and of cathepsin B was reversed virtually completely within 24 h, when the inhibitor was removed from the medium. Since the inhibitor forms a covalent bond with the enzyme, the recovery of cathepsin B activity presumably reflects production of new molecules of active enzyme. 5. The inhibitory effects of pepstatin, the carboxyl proteinase inhibitor, were under some circumstances additive with those of Z-Phe-Ala-CHN₂, and were also largely reversible. 6. It is concluded that thiol proteinases play a major role in lysosomal proteolysis in cultured macrophages.

Evidence has accumulated which indicates that lysosomes are involved in the degradation of intracellular proteins in many cells (Dean, 1975; Dean & Barrett, 1976; Goldberg & St. John, 1976; Amenta *et al.*, 1977, 1978; Ballard, 1978). Much of the evidence has concerned inhibition of degradation by various agents such as proteinase inhibitors, in particular pepstatin, directed to the lysosomal system (Dean, 1975). In most experimental work the organ or cells concerned have been studied under conditions of nutritional and hormonal deprivation in which proteolysis is 'accelerated' above the 'basal' rates which obtain in nutritionally and hormonally supplemented conditions (see Goldberg & St. John, 1976). Several authors have therefore argued that lysosomes may be involved only in the 'accelerated' proteolysis. However, Dean (1979) has presented some work on protein degradation in cultured mouse macrophages in which pepstatin, entering the cells

by pinocytosis, was shown to inhibit even basal proteolysis: this supports the idea that lysosomes participate in both basal and accelerated degradation.

Until recently, pepstatin seemed to be the only proteinase inhibitor with sufficient group-specificity and effectiveness to allow the demonstration of participation of proteinases of particular catalytic groups in intracellular proteolysis. Thus previous conclusions on lysosomal proteolysis have largely concerned the participation of carboxyl proteinases, particularly cathepsin D (susceptible to pepstatin). However, thiol proteinases are possibly even more potent than carboxyl proteinases in lysosomal proteolysis (see Barrett, 1977). Therefore the present paper describes the effects on macrophage basal proteolysis of a new synthetic inhibitor, Z-Phe-Ala-CHN₂, which is selectively active on thiol proteinases. The inhibition of proteolysis is correlated with the inhibition of intracellular cathepsin B.

Materials and Methods

Materials for cell culture

Multi-well Linbro culture dishes (2 cm²) were from Flow Laboratories, Irvine, Scotland, U.K., and cul-

Abbreviation used: Z-Phe-Ala-CHN₂, benzyloxycarbonylphenylalanyldiazomethane (benzyloxycarbonylphenylalanylalanine diazomethyl ketone).

* Present address: Department of Biology, Brookhaven National Laboratory, Upton, NY 11973, U.S.A.

† To whom reprint requests should be addressed. Present address: Department of Applied Biology, Brunel University, Uxbridge, Middx. UB8 3PH, U.K.

ture media and sera were obtained from Bio-Cult Laboratories, Glasgow, Scotland, U.K.

Biochemical reagents

Penicillin and streptomycin were supplied by Sigma Chemical Co., Poole, Dorset, U.K. Heparin (preservative-free) was from Paines and Byrne, Greenford, Middx, U.K. L-[U-¹⁴C]Leucine was from The Radiochemical Centre, Amersham, Bucks., U.K. Pepstatin was obtained from the Protein Research Foundation, 476, Ina, Minoh-Shi, Osaka 562, Japan. It was dissolved in methanol at 10mg/ml, and was diluted from this stock into media at the specified concentration. Methanol was used at a maximum concentration of 1% (v/v) in the cultures and had no effect on proteolysis, although it slightly accelerated cell death.

Z-Phe-Ala-CHN₂ was synthesized (Watanabe *et al.*, 1979) by the method described for an analogous reagent (Leary & Shaw, 1977). A radioactive form of this inhibitor was also prepared by using ¹⁴C-labelled diazomethane (Leary *et al.*, 1977), and had sp. radioactivity 5.89×10^6 d.p.m./ μ mol. Both samples were kind gifts from Dr. H. Watanabe and Dr. G. Green (Department of Biology, Brookhaven National Laboratory, Upton, NY, U.S.A.). For use with macrophages, a stock solution of 50mM inhibitor in dimethyl sulphoxide was prepared, and diluted 1:500 with medium to provide a final concentration of 0.1mM. This was the highest concentration of inhibitor studied. The resultant dimethyl sulphoxide concentration (0.2%, v/v) was shown to have no effect on protein turnover or cell death. The stock solution of inhibitor was stored at 4°C, but not for longer than 2 weeks. In experiments involving combinations of pepstatin and Z-Phe-Ala-CHN₂, both inhibitors were made up in methanol, which was maintained at 1% (v/v) in both control cultures and those containing inhibitors. Z-Phe-Ala was obtained from Sigma. Partially purified cathepsin D and highly purified cathepsin B, both from human liver, were kind gifts from Dr. A. J. Barrett (Strangeways Research Laboratory, Cambridge, U.K.).

Assays

Papain was measured by an esterase assay (Leary *et al.*, 1977). Cathepsin D was measured by method 1 described by Barrett & Heath (1977), at 37°C, with haemoglobin as substrate. One unit of enzyme causes an increase in A_{280} of the trichloroacetic acid-soluble materials of 1, in a 1h incubation under assay conditions. Cathepsin B was assayed against benzyloxycarbonylarginylarginine 2-naphthylamide, followed by Fast Garnet GBC coupling of the product (Barrett, 1972). We are grateful to Dr. C. G. Knight (Strangeways) for a sample of this substrate. Protein was measured by the procedure of

Lowry *et al.* (1951), with bovine serum albumin as standard.

Macrophage collection and culture

The methods employed have been described in detail previously (Dean, 1979). The cells (from normal TO mice) were cultivated in Medium 199 containing 10% (v/v) heat-inactivated acid-treated foetal calf serum, with 100 international units of penicillin/ml and 100 μ g of streptomycin/ml. The acid treatment (pH 3, 30min) was used to destroy some proteinase inhibitors (Werb & Gordon, 1975). After establishment of the cultures overnight (in 2cm² Linbro culture wells, with 2ml of medium) the medium was changed to leucine-free modified Eagle's Medium with 10% (v/v) heat-inactivated acid-treated foetal calf serum, containing 0.1 μ Ci of L-[U-¹⁴C]leucine/ml. After 24h, the cells were washed four times with phosphate-buffered saline (comprising 136mM-NaCl, 2.7mM-KCl, 8.1mM-Na₂HPO₄ and 1.5mM-KH₂PO₄, without Ca²⁺ and Mg²⁺), containing 10mM-leucine. This washing procedure was found to deplete the cells satisfactorily of radioactive low-molecular-weight substances such as free amino acids; less than 4% of the radioactivity in the cells was found to be soluble in 5% (w/v) trichloroacetic acid. The macrophages then received fresh modified Eagle's medium with serum as before, and with 10mM-leucine (unlabelled). This concentration of leucine has been shown previously to suppress reutilization of radioactive leucine (Dean, 1979). Inhibitors or solvents were added to the medium as indicated in the Results section. All experiments were performed with at least triplicate cultures, and were repeated several times. After various periods of further culture, medium was removed for measurement of radioactivity; 0.5ml was treated with 0.5ml of 10% trichloroacetic acid/100mM-leucine. After centrifugation (200g for 5min), 0.5ml of the supernatant was removed for radioactivity counting. The precipitate was washed twice with 5% trichloroacetic acid/10mM-leucine, and dissolved in 1ml of 99% formic acid; 0.5ml of the solution was removed for radioactivity counting.

The cells remaining on the plate were scraped off with two 0.5ml portions of 0.1% (v/v) Triton X-100, unless cathepsin B was to be measured. In that case 50mM-sodium acetate buffer, pH 5.0, was used, since cathepsin B is unstable at neutral pH (Barrett, 1977). For radioactivity counting, 0.5ml of extract, plus 50 μ l of 10% bovine serum albumin, were treated with 0.5ml of 10% trichloroacetic acid/100mM-leucine, as described above for the medium, and the processing was continued to provide samples of the cellular trichloroacetic acid-soluble and insoluble fractions for radioactivity counting.

Standards were counted for radioactivity in

aqueous trichloroacetic acid and in formic acid to measure counting efficiency for appropriate correction. A Triton X-100/toluene scintillant (3:7, v/v) was used. Results are expressed as percentage degradation ($\pm$ S.D.), which is computed as trichloroacetic acid-soluble radioactivity as a percentage of total radioactivity in the system. Trichloroacetic acid-precipitable material in the medium gave an upper estimate of the amount of cell death occurring in the cultures. All the data quoted in the Results section are from experiments conducted with triplicate observations for each condition.

Results and Discussion

The major proteolytic enzymes responsible for lysosomal protein degradation are probably thiol proteinases (such as cathepsins B and L) and carboxyl proteinases such as cathepsin D (see Barrett, 1977). In establishing the group selectivity of peptidyl diazomethyl ketones for the inactivation of thiol proteinases, a lack of action on chymotrypsin, a representative serine proteinase, had been shown (H. Watanabe, G. Green & E. Shaw, unpublished work). However, a possible effect on cathepsin D, a representative carboxyl proteinase, had not been examined. For this purpose cathepsin D (9 units/ml) was incubated at 37°C with 50 μ M-Z-Phe-Ala-CHN₂ at pH 5.0 for 1 h. No loss of ability to digest haemoglobin was observed beyond that of the control sample in the same solvent (20% dimethyl sulphoxide). This loss of activity against haemoglobin was always less than 10%.

In preparation for its use under conditions of cell culture, the stability of Z-Phe-Ala-CHN₂ was examined. The inhibitor was incubated in complete medium at 10 μ M at 37°C. Samples were diluted to 0.1 μ M and examined for ability to inactivate papain. It was estimated that incubation for 2 days resulted in a minor loss of activity, about 15%. For use with macrophages, therefore, replenishment of medium with fresh inhibitor was not considered necessary.

Various concentrations of Z-Phe-Ala-CHN₂ were added to cultures of macrophages which had been incubated with [¹⁴C]leucine as described in the Materials and Methods section, and the effect on protein turnover was examined. Inhibition of protein turnover was consistently observed when Z-Phe-Ala-CHN₂ was present in the range 10–100 μ M, and was dose-dependent (Fig. 1). At 100 μ M, after 24 h, inhibition of protein degradation generally reached 20–35% (Table 1, Expt. 1b), depending on the batch of macrophages. Even at earlier times such as 6–10 h (Table 1, Expts. 1a and 2), some inhibition was evident, and it increased in individual experiments as the duration of exposure was increased, up to a maximum at which it remained roughly constant for some time (see Table 2). Pepstatin, the group-

Fig. 1. Dose dependence of inhibition of macrophage protein degradation by Z-Phe-Ala-CHN₂.

Prelabelled cells were allowed to degrade protein for 24 h in the presence of the specified concentrations of inhibitor. All culture media contained 0.2% dimethyl sulphoxide. ●, Protein degradation (% of total prelabelled protein); ○, inhibition of degradation (%).

specific inhibitor for carboxyl proteinases (see Umezawa & Aoyagi, 1977), was examined in combination with 100 μ M-Z-Phe-Ala-CHN₂ to determine whether an increased inhibitory effect resulted. At 20 μ g/ml, pepstatin did not in these experiments have a statistically significant effect on protein degradation by itself, but it enhanced the action of Z-Phe-Ala-CHN₂ whether measured at 6 h (Table 1, Expt. 2) or 21 h (Table 1, Expt. 3). At a higher concentration, 100 μ g/ml, pepstatin did inhibit protein degradation (as shown earlier; Dean, 1979), and the combination was again more effective (Table 1, Expt. 4). The variability of susceptibility of the macrophages to inhibition by the added inhibitors has been discussed previously (Dean, 1979).

In view of the inhibition of protein turnover in macrophages incubated in Z-Phe-Ala-CHN₂, it was decided to examine the intracellular thiol proteinase activity in the hope of correlating the observed metabolic effect with the actual enzyme activity. Initial attempts to measure the kinetics of inactivation gave the impression that the process was proceeding very rapidly. For example, when cells were exposed to 100 μ M-inhibitor for 2 min, washed four times with phosphate-buffered saline, and extracts assayed for cathepsin B activity, only 8% of the activity in control cultures could be recovered. It was appreciated that free inhibitor on the surface of the cells, or internalized but not accessible to the lysosomal contents, might inactivate cathepsin B during the rupture of the cells and the long assay (about 20 h) required because of the low enzyme activities available for study. Some indication of this was seen in the fact that, with accelerated cell processing and increased substrate concentration (1.5-fold) in the assay allowing a briefer incubation, an estimate of 64% inactivation after a 5 min exposure to the inhibitor could be made, in contrast with the estimate of about 90% made with normal techniques.

A radioactive form of the inhibitor was synthe-

Table 1. *Effect of Z-Phe-Ala-CHN₂ on macrophage protein degradation alone (Expt 1) or with pepstatin (Expts. 2–4)* Prelabelled cultures were incubated for the stated times in the presence of Z-Phe-Ala-CHN₂, pepstatin or both inhibitors; 1% methanol was present as solvent in all cultures.

Expt.	Conditions	Time (h)	Percentage of total radioactivity solubilized	Inhibition (%)
1(a)	Control	10	19.3 ± 0.6	0
	100 μM-Z-Phe-Ala-CHN ₂		14.9 ± 1.1	23.1
	50 μM-Z-Phe-Ala-CHN ₂		16.7 ± 2.4	14.0
1(b)	Control	24	32.0 ± 3.2	0
	100 μM-Z-Phe-Ala-CHN ₂		22.2 ± 3.5	32.4
	50 μM-Z-Phe-Ala-CHN ₂		24.7 ± 1.9	22.7
2	Control	6	31.3 ± 1.3	0
	100 μM-Z-Phe-Ala-CHN ₂		27.7 ± 0.5	11.5
	Pepstatin (20 μg/ml)		30.9 ± 1.5	(1.3)
	Both inhibitors		25.6 ± 0.5	18.2
3	Control	21	43.8 ± 1.9	0
	100 μM-Z-Phe-Ala-CHN ₂		32.2 ± 1.2	26.5
	Pepstatin (20 μg/ml)		42.9 ± 2.6	(2.1)
	Both inhibitors		28.6 ± 0.7	34.7
4	Control	21	50.3 ± 2.1	0
	100 μM-Z-Phe-Ala-CHN ₂		38.2 ± 1.5	24.1
	Pepstatin (100 μg/ml)		42.8 ± 0.2	14.9
	Both inhibitors		35.1 ± 0.4	30.2

Table 2. *Inhibition of protein degradation in cultured mouse macrophages by Z-Phe-Ala-CHN₂* Experimental conditions were as described in Table 1 and the Materials and Methods section; dimethyl sulphoxide (0.2%, v/v) was present in all cultures during degradation, as solvent.

Duration of degradation (h)	Experimental conditions	Degradation of original labelled protein (%)	Inhibition of degradation (%)
6	Control	13.0 ± 0.7	—
	10 μM-Z-Phe-Ala-CHN ₂	11.6 ± 1.3	10.7
	100 μM-Z-Phe-Ala-CHN ₂	10.8 ± 0.6	16.9
10	Control	20.3 ± 0.6	—
	10 μM-Z-Phe-Ala-CHN ₂	15.8 ± 2.9	22.1
	100 μM-Z-Phe-Ala-CHN ₂	15.8 ± 1.1	21.8
24	Control	32.9 ± 3.2	—
	10 μM-Z-Phe-Ala-CHN ₂	25.8 ± 1.3	21.6
	100 μM-Z-Phe-Ala-CHN ₂	23.2 ± 3.4	29.5

sized, namely Z-Phe-Ala-[¹⁴C]CHN₂, to help gain some insight into the process of inhibitor uptake. When macrophages were exposed to 100 μM-inhibitor containing 5.89×10^6 d.p.m./μmol, there was an association of 84 pmol/100 μg of cellular protein within 5 min, and this did not significantly change during the first 1 h, but increased to 169 pmol at 6 h and 305 pmol at 24 h. To examine the possibility that some proportion of the inhibitor was adsorbed on the surface of the cells, after five washes of the cells with phosphate-buffered saline, non-radioactive inhibitor at 100 μM was added to the phosphate-buffered saline for a six wash of the cells. The radioactivity appearing in the wash was double that of a sixth wash without inhibitor, indicating a displaceable population of inhibitor molecules.

In order to remove this adsorbed inhibitor, a washing procedure was developed, using the dipeptide derivative Z-Phe-Ala. Macrophages were exposed to labelled inhibitor (100 μM) for 5 min. The cultures were then washed four times, with a 5 min incubation at 37°C for each washing. Three different washing media were compared: phosphate-buffered saline, culture medium, and culture medium containing 1 mM-Z-Phe-Ala. Far more radioactivity was displaced by medium containing Z-Phe-Ala than by the other washing media, and by the fifth wash with Z-Phe-Ala the radioactivity removed was not significantly above background, whereas significant amounts of radioactivity were still appearing in the other washing fluids. After four washes with phosphate-buffered saline, culture medium or

culture medium containing 1 mM-Z-Phe-Ala, the amounts of inhibitor left associated with the cells (per 100 μ g of cell protein) were respectively 45 pmol, 35 pmol and 13 pmol. Washing with Z-Phe-Ala was found to be as efficient in serum-free medium as in serum-containing medium. Therefore, for subsequent experiments on uptake of inhibitor and on the kinetics of inhibition of intracellular cathepsin B, four washes with 1 mM-Z-Phe-Ala in serum-free medium, with 5 min at 37°C for each wash, were used.

By this procedure it was established that radioactivity fixed to the cells, presumably mainly internalized, represented 13 pmol/100 μ g of cell protein in the early minutes, progressing gradually to 49 pmol/100 μ g of cell protein after 18 h. The slow-uptake phase is suggestive of entry by pinocytosis, rather than permeation, which is normally a more rapid process. It is very difficult to prove this conclusively. However, at 4°C the slow uptake of inhibitor is virtually completely suppressed, as is pinocytosis. Thus internalized inhibitor is probably present in endocytic vesicles, and after fusion of these vesicles with lysosomes (see Dean & Barrett, 1976) in secondary lysosomes (as is also indicated by the observed inhibition of cathepsin B; see below).

Thus approx. 50 pmol of Z-Phe-Ala-CHN₂/100 μ g of cell protein was firmly associated with the cells after 24 h. Because of the small amount of uptake, and its likely predominant localization in lysosomes, it is unlikely that general effects on extra-lysosomal metabolism were induced. The endocytic index for uptake (see Pratten *et al.*, 1978) was 0.02–0.05 μ l/h per 10⁶ cells. Assuming that 10⁶ macrophages have a total volume of 1 μ l (Pofit & Strauss, 1977), and that lysosomes occupy 1% of that volume (see discussion in Dean, 1977), the intra-lysosomal concentration of Z-Phe-Ala-CHN₂ might attain several mM by 24 h. It remains possible that

some inhibitor molecules might be lost by inactivation. Exocytosis of free inhibitor is unlikely, because of the covalent nature of the bond formed between it and thiol proteinases, but exocytosis of enzyme-inhibitor complexes could conceivably occur. Because of these complications, the estimate of intralysosomal concentration must be taken as very approximate.

Removal of the adsorbed inhibitor from macrophages by washes with 1 mM-Z-Phe-Ala as described now permitted a more accurate determination of the rate of inactivation of cathepsin B intracellularly. After exposure to 100 μ M-Z-Phe-Ala-CHN₂ for 5, 30, 60 and 90 min and washing with Z-Phe-Ala, the macrophage content of cathepsin B fell to 72, 40, 25 and 5% of control respectively. A half-time of inactivation of about 25 min was estimated to result on incubation of macrophages with this concentration of inhibitor.

In the studies described above on the inhibition of protein turnover by Z-Phe-Ala-CHN₂, the inhibitor was introduced at the beginning of the degradative period. Under these conditions cathepsin B continues to function until completely inactivated by arrival of inhibitor, and the observed inhibition may not adequately reflect the contribution of cathepsin B to protein turnover. An additional experiment was carried out with Z-Phe-Ala-CHN₂ present at 100 μ M during the prelabelling phase, so that, on transfer to medium permitting measurement of protein degradation, the macrophages had no active cathepsin B. The presence of inhibitor in the latter medium maintained this state, but in pretreated cultures which were then placed in control medium proteolysis reverted to higher values. Z-Phe-Ala-CHN₂ inhibited degradation in pretreated cultures to a slightly greater degree than in cultures prelabelled normally (30.7 versus 26.1% inhibition, respectively; Table 3, Expt. 2, control). The pretreated cul-

Table 3. Inhibition of macrophage protein degradation by Z-Phe-Ala-CHN₂ plus pepstatin: effect of prelabelling proteins in the presence of Z-Phe-Ala-CHN₂

Macrophage cultures were prelabelled for 24 h with L-[U-¹⁴C]leucine (0.1 μ Ci/ml) either in normal medium or in medium containing 100 μ M-Z-Phe-Ala-CHN₂. After labelling, the medium was removed and was replaced by normal degradation medium with the additions indicated under 'Degradation conditions'. Methanol was maintained at 1% (v/v) in all cultures during both the prelabelling and the degradation period of 21 h.

	Degradation conditions	Percentage of total radioactivity solubilized	Inhibition (%)
1. Normal prelabelling	Control	31.0 $\pm$ 1.6	0
	Z-Phe-Ala-CHN ₂ (100 μ M)	22.9 $\pm$ 0.8	26.1
	Pepstatin (100 μ g/ml)	29.2 $\pm$ 1.6	5.8
	Both inhibitors	21.3 $\pm$ 0.1	31.3
2. Prelabelling in 100 μ M-Z-Phe-Ala-CHN ₂	Control	37.8 $\pm$ 1.8	0
	Z-Phe-Ala-CHN ₂ (100 μ M)	26.2 $\pm$ 0.0	30.7
	Pepstatin (100 μ g/ml)	32.3 $\pm$ 3.4	14.6
	Both inhibitors	21.3 $\pm$ 0.9	43.7

tures actually degraded their labelled protein faster than the control cultures that had been prelabelled in the absence of inhibitor. This presumably indicates that the cells prelabelled in the presence of inhibitor accumulate labelled proteins which can then be very rapidly degraded when cathepsin B activity is recovered, as in the control group of Expt. 2, Table 3. Virtually complete recovery of cathepsin B activity was independently confirmed under these conditions. The effects of pepstatin and Z-Phe-Ala-CHN₂ were additive as before, and again were more pronounced when cells were prelabelled in the presence of Z-Phe-Ala-CHN₂.

In other experiments cells were prelabelled in the presence of pepstatin (100 µg/ml), or in the presence of both pepstatin (100 µg/ml) and Z-Phe-Ala-CHN₂ (100 µM). When such pretreated cultures were then allowed to degrade protein in the virtual absence of inhibitors (medium changed to fresh control medium), degradation proceeded at similar rates to that in cultures which were prelabelled normally in the absence of inhibitors. Cathepsin D activity also recovered to about 75% of control during the 24 h degradation period. This was determined by assaying cathepsin D in cultures exposed to pepstatin for 24 or 48 h, in cultures preincubated for 24 h in pepstatin and then incubated for a further 24 h in the absence of extracellular inhibitor (medium changed at 24 h), and in controls for each time point. When retained during the degradation period, pepstatin and Z-Phe-Ala-CHN₂ suppressed proteolysis, and their effects were again approximately additive, up to a maximum inhibition of 35% (compared with pretreated cells degrading in the absence of inhibitors). It should be noted that the protein labelling in the presence of inhibitors may be quantitatively or even qualitatively different from that in normally labelled cultures.

These experiments showed that the effects of pepstatin and Z-Phe-Ala-CHN₂ were reversible. As Z-Phe-Ala-CHN₂ forms covalent complexes with thiol proteinases, it may be deduced that recovery of cathepsin B activity results from the production of new molecules of active cathepsin B. Although the effects of the two inhibitors were additive in some conditions, the degree of inhibition obtained was never greater than 45%, in spite of the pretreatment with inhibitor. Because Z-Phe-Ala-CHN₂ seems to enter macrophages by pinocytosis (as does pepstatin; Dean, 1979), its effects are almost certainly exerted on lysosomal proteolysis. Thus the present

evidence supports and extends previous work indicating lysosomal participation in macrophage basal proteolysis, and specifically implicates thiol proteinases. Although we have demonstrated inhibition of the enzyme active on Z-Arg-Arg 2-naphthylamide, primarily cathepsin B, it is likely that other thiol proteinases, such as cathepsin L (Kirschke *et al.*, 1977), are affected also. The maximum inhibition observed, 45%, suggests that a maximum of 55% of macrophage basal proteolysis is non-lysosomal. But, as inhibition of one pathway of degradation may accelerate another, this may well be a considerable overestimate of non-lysosomal proteolysis.

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