Biochem. J. (1981) **196**, 41–48
Printed in Great Britain

Effects of microbial proteinase inhibitors on the degradation of endogenous and internalized proteins by rat yolk sacs

Spencer E. KNOWLES and F. John BALLARD*
CSIRO Division of Human Nutrition, Adelaide, S.A. 5000, Australia

and Geoffrey LIVESEY and Kenneth E. WILLIAMS
Department of Biological Sciences, University of Keele, Keele, Staffs. ST5 5BG, U.K.

(*Received 5 September 1980/Accepted 29 December 1980*)

1. The effects of leupeptin and other microbial proteinase inhibitors were measured in rat yolk sacs on the uptake and degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin as well as on the degradation of ^{3}H-labelled endogenous protein. 2. Leupeptin, at concentrations between 1 and 100 μg/ml, inhibits the degradation of added albumin without affecting pinocytic uptake. Accordingly large amounts of undegraded albumin accumulate within the tissue. 3. Removal of leupeptin produces a rapid recovery of the capacity to degrade albumin. 4. Endogenous protein degradation is rapidly inhibited by leupeptin, but to a far lesser extent than the breakdown of albumin. However, the inhibition is only slightly reversed on removal of leupeptin. 5. Degradation of both albumin and endogenous protein in intact yolk sacs is inhibited by the microbial proteinase inhibitors in the order: leupeptin > antipain > chymostatin; elastatinal, pepstatin and bestatin are ineffective. 6. Similar results are found when albumin is incubated in yolk-sac homogenates at pH 4 with the inhibitors. 7. The marked inhibitory effects of leupeptin, antipain and chymostatin suggest that cathepsin B and possibly cathepsin L participate in the degradation of ^{125}I-labelled albumin in yolk sacs. By comparison, the smaller inhibitory effects of the proteinase inhibitors on endogenous protein breakdown imply a minor role of lysosomal cathepsins in this process.

Ammonia and other weak bases inhibit the degradation of ^{3}H-labelled endogenous proteins in the rat yolk sac to a far lesser extent than the breakdown of pinocytically captured formaldehyde-denatured ^{125}I-labelled bovine serum albumin (Livesey *et al.*, 1980). Such experiments provide evidence that endogenous proteins are only degraded in part by a lysosomal system requiring an acidic environment, in support of other studies with cultured cells (Knowles & Ballard, 1976; Amenta *et al.*, 1977; Ballard, 1977; Hopgood *et al.*, 1980). However, studies with yolk sacs show that weak bases are not entirely satisfactory as probes to detect selective effects, because they inhibit endocytosis and also decrease the phosphorylation state of adenine nucleotides (Livesey *et al.*, 1980). To overcome these problems we have tested several of the microbial proteinase inhibitors that have been described by Umezawa (1977). Since these inhibitors are effective at low concentrations, they might not be expected to interfere with other cell functions. Our results show that the catabolism of ^{125}I-labelled albumin is inhibited by the cathepsin B inhibitor, leupeptin, to a much greater extent than is the breakdown of endogenous proteins, whereas pinocytosis is not affected. The experiments provide further evidence against a wholly lysosomal site of degradation for endogenous proteins.

* To whom reprint requests should be addressed.

Materials and methods

Materials

Leupeptin, pepstatin, antipain, elastatinal, bestatin, chymostatin and phosphoramidon were either kindly provided by Dr. H. Umezawa, Microbial Chemistry Research Foundation, 14–23, Kamiosaki 3-Chome, Shinagawa-Ku, Tokyo, Japan, or were a gift from Dr. W. Troll, Department of Environmental Medicine, New York University Medical Center, New York, U.S.A. Other materials were as described by Livesey *et al.* (1980).

0306-3283/81/040041-08$01.50/1

Rates of uptake and degradation of ^{125}I-labelled albumin

Each proteinase inhibitor was added to 20ml of medium 199 together with a single yolk sac. After 30min preincubation at 37°C, ^{125}I-labelled albumin (20μg) was added. Sequential samples of medium were taken over a 3h period and rates of uptake and degradation expressed as ng of ^{125}I-labelled albumin/mg of yolk-sac protein per h. These methods are essentially as described by Livesey & Williams (1979). Slight differences between batches are found in the rate of capture of ^{125}I-labelled albumin by yolk sacs. This occurs because denaturation conditions required for the preparation of ^{125}I-labelled albumin cannot be reproduced exactly. Recovery of the capacity to degrade albumin was measured after 1h exposure to leupeptin. For this purpose each sac was rinsed three times in medium 199 (approx. 30ml) before being placed in fresh medium 199 containing labelled substrate. Uptake, degradation and accumulation of albumin were measured in separate yolk sacs at different times after removal of leupeptin.

Degradation of ^{3}H-labelled endogenous protein

The degradation of endogenous yolk-sac protein was measured as described previously (Livesey *et al.*, 1980). Briefly, sacs were incubated for 3h in Dulbecco-modified Eagle's Minimal Essential Medium containing 2μCi of [^{3}H]leucine/ml and 10μM-L-leucine, rinsed in radioisotope-free medium and incubated for a further 90min in 'degradation' medium [Eagle's Minimal Essential Medium containing 2mM-L-leucine and 20mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid, pH 7.5)] to degrade unstable proteins. The sacs were subsequently transferred to fresh 'degradation' medium containing the proteinase inhibitor and incubated, usually for 4h. Rates of degradation were expressed as the percentage of ^{3}H-labelled protein that was degraded over this period.

The time course of leupeptin inhibition was followed by measuring the percentage of cell protein degraded in separate yolk sacs after 1, 2, 3 and 4h incubation. In studies of recovery from leupeptin inhibition (see Fig. 4) the sacs were removed from the medium after 2h exposure to leupeptin or to control medium, rinsed three times and incubated for up to 2h more in fresh medium containing no leupeptin. The percentage of protein degraded after 3 and 4h was calculated by summing the amount degraded in the 1 or 2h reincubation periods and the appropriate amounts calculated from data obtained with separate yolk sacs incubated for the initial 2h.

Measurements of yolk-sac viability

Yolk sacs were labelled with [^{3}H]leucine as described for the measurement of endogenous protein breakdown, and the percentage of trichloroacetic acid-insoluble radioactivity appearing in the medium during a 4h incubation was taken as a measure of protein loss from damaged cells. This determination was carried out as a viability control in all measurements of endogenous protein degradation.

Release of lactate dehydrogenase was followed during incubation of yolk sacs under similar conditions to those used for measuring the degradation of endogenous proteins. Lactate dehydrogenase was measured as described by Kornberg (1955).

Yolk sacs, incubated for 2h under the same conditions as used for measurement of endogenous protein degradation, were freeze-clamped and extracted with $HClO_4$ (Livesey *et al.*, 1980). The extracts were neutralized for the determination of ATP, ADP and AMP (Ballard, 1971).

Results

Effects of leupeptin on the uptake and degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin

Preliminary experiments showed that leupeptin, added at a concentration of 20μg/ml to medium 199, inhibited the degradation of ^{125}I-labelled albumin by yolk sacs. Fig. 1 shows the time-course of

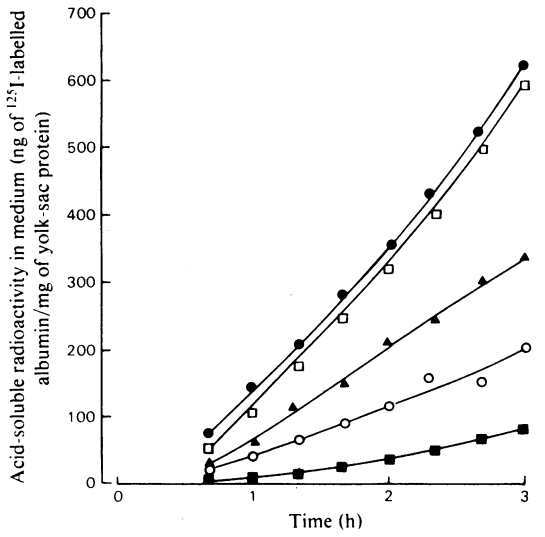

Fig. 1. *Time course of degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by yolk sacs in the presence of leupeptin*

Individual yolk sacs were preincubated in 20ml of medium with leupeptin for 30min before the addition of 20μg of ^{125}I-labelled albumin. Degradation is expressed as the amount of denatured albumin converted into trichloroacetic acid-soluble material. The leupeptin concentrations (μg/ml) were: ●, 0; □, 1; ▲, 20; ○, 40; and ■, 100.

the appearance of acid-soluble radioactivity in the incubation medium when yolk sacs were incubated with ^{125}I-labelled albumin in the presence of different concentrations of leupeptin. At each leupeptin concentration, the trichloroacetic acid-soluble radioactivity appearing in the medium, after an initial lag period, increased essentially linearly with time over the remainder of the 3 h incubation period.

The effects of the different concentrations of leupeptin on the rates of uptake and of degradation of ^{125}I-labelled albumin and on its accumulation by yolk sacs are shown in Fig. 2. A small inhibition of uptake was observed in the presence of leupeptin at 100μg/ml (Fig. 2*a*), but this was not statistically significant ($P<0.10$). As the leupeptin concentration was increased, the rate of degradation of the ^{125}I-labelled albumin progressively decreased, reaching about 20% of the control rate in the presence of 100μg of leupeptin/ml. The decreased rate of degradation was accompanied by an increase in the quantity of radioactivity associated with the tissue after 3 h incubation (Fig. 2*b*). For incubations in medium containing 40μg of leupeptin/ml the total amount of tissue radioactivity reached a maximum value equivalent to 4 times that in control yolk sacs. The amount of acid-soluble radioactivity in the yolk-sac tissue remained approximately constant at 34–50 ng/mg of yolk-sac protein, irrespective of the leupeptin concentration.

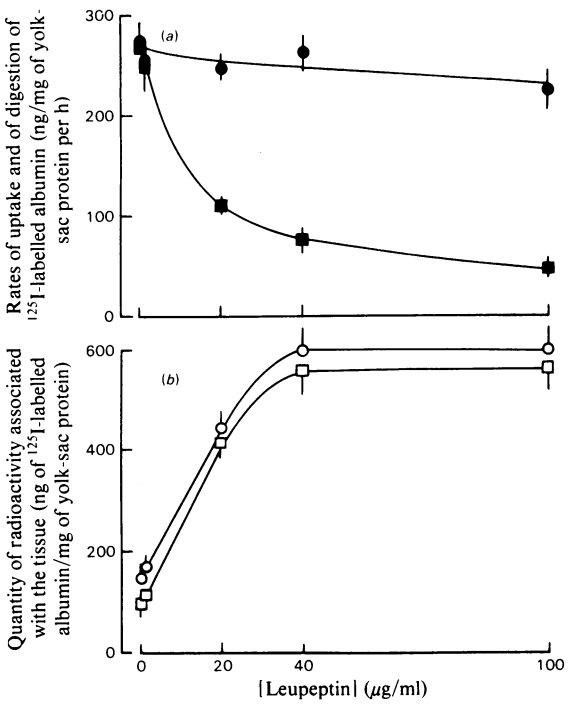

Fig. 2. *Effects of leupeptin concentration on the uptake, accumulation and degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by yolk sacs*

Yolk sacs were incubated as described in the text and the legend to Fig. 1 for the measurement of (*a*) the rates of albumin uptake (●) and degradation (■). These rates were expressed as ng/mg of yolk-sac protein per h. At the end of 3 h incubation, (*b*) the total tissue-associated radioactivity (○) and the amount of this radioactivity precipitated with trichloroacetic acid (□) were measured and expressed as ng/mg of yolk-sac protein. Values at each leupeptin concentration are means ± S.E.M. for four determinations.

Yolk sacs exposed to 40μg of leupeptin/ml of medium for 1 h showed a rapid recovery of the majority of their proteolytic activity after being washed and re-incubated in inhibitor-free medium 199 containing ^{125}I-labelled albumin (Table 1). A slow, progressive recovery of the remainder of the proteolytic activity occurred over longer washing periods, but complete recovery was not observed even after 2 h of incubation in leupeptin-free medium. In the control experiments yolk sacs maintained a constant degradative capacity relative to substrate uptake throughout the duration of the experiment.

Inhibition of endogenous protein degradation by leupeptin

Leupeptin inhibits the degradation of [^{3}H]-leucine-labelled endogenous yolk-sac proteins (Fig. 3). The effect increases with increasing leupeptin concentration, but even at 100μg of leupeptin/ml degradation occurs at about 80% of the corresponding control rate. A more detailed investigation of the time course of leupeptin action is shown in Fig. 4.

In these experiments inhibition is detectable after 1 h of incubation in the presence of 100μg of leupeptin/ml of medium and reaches 17% by 2 h. The percentage effect does not increase as the exposure period is extended to 4 h, suggesting a rapid but not a progressive response.

After 2 h exposure to leupeptin some yolk sacs were rinsed three times in leupeptin-free medium and incubated in either the presence or the absence of the proteinase inhibitor (Fig. 4). Although a slight recovery of degradation rate was detectable after 1 h or 2 h further incubation, this increased rate was still below that found in yolk sacs never exposed to leupeptin.

Viability of yolk sacs exposed to leupeptin

Yolk sacs incubated under control conditions release small amounts of lactate dehydrogenase into the medium. In agreement with Livesey *et al.* (1980), the amount released over 4 h represents less than 2% of the total enzyme content of yolk sacs and is not increased by leupeptin (Table 2). Labelled

Table 1. *Recovery of proteolytic activity towards ^{125}I-labelled albumin by yolk sacs previously exposed to leupeptin*
Yolk sacs were either incubated continuously in medium containing leupeptin (40 μg/ml) or incubated under such conditions for 1 h before being rinsed and then reincubated in leupeptin-free medium for 8, 60 or 120 min periods. After these washout times, 1 μg of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (^{125}I-BSA) was added per ml of medium, and rates of uptake and degradation by the tissue were determined over a further 3 h period. By expressing the rate of degradation as a percentage of the rate of uptake, any net loss in proteolytic capacity of the tissue is readily apparent. Equivalent data were obtained with tissues incubated for the same periods, but in the absence of leupeptin. The values shown are means (±S.E.M.) for four determinations, with corresponding data from matched controls shown in square brackets.

Conditions before adding ^{125}I-BSA		Degradation of ^{125}I-BSA by yolk sacs exposed to leupeptin and by matched controls not exposed to leupeptin		
Period of exposure to leupeptin	Incubation period in leupeptin-free medium	Rate of degradation of ^{125}I-BSA (% of rate of uptake)	Proportion of tissue radioactivity that is acid-soluble (%)	Proportion of ingested ^{125}I-BSA retained by the tissue at 3 h (%)
Continuous	—	27.1 ± 9.7 [99.6 ± 1.1]	8.6 ± 1.2 [35.3 ± 3.2]	75.3 ± 6.9 [22.1 ± 2.4]
1 h	8 min	75.4 ± 5.7 [95.7 ± 2.8]	17.8 ± 2.9 [37.3 ± 4.2]	43.3 ± 3.6 [19.5 ± 2.5]
1 h	1 h	79.5 ± 4.1 [98.1 ± 1.6]	19.8 ± 1.7 [37.8 ± 2.8]	37.9 ± 2.8 [19.6 ± 0.8]
1 h	2 h	83.1 ± 5.9 [95.8 ± 1.4]	23.9 ± 1.7 [33.8 ± 2.1]	34.3 ± 4.2 [19.7 ± 0.9]

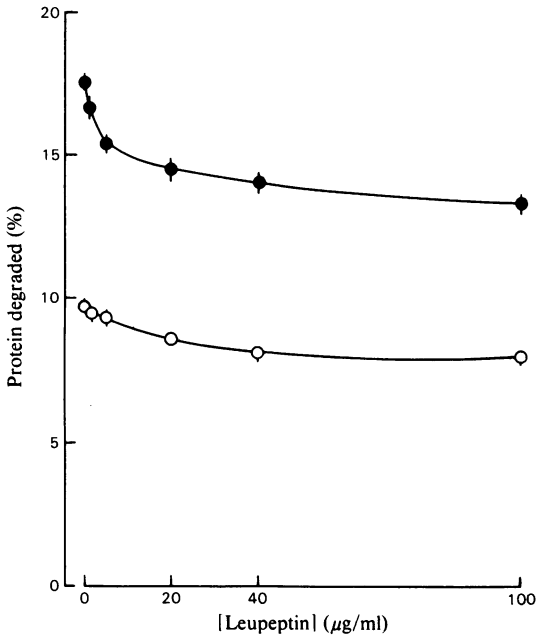

Fig. 3. *Effect of leupeptin on the degradation of [^3H]leucine-labelled endogenous protein*
The percentage of labelled protein degraded is reported after 2 h (○) and 4 h (●) incubation in the presence of the indicated leupeptin concentrations. Values are means ± S.E.M. for ten determinations at each leupeptin concentration.

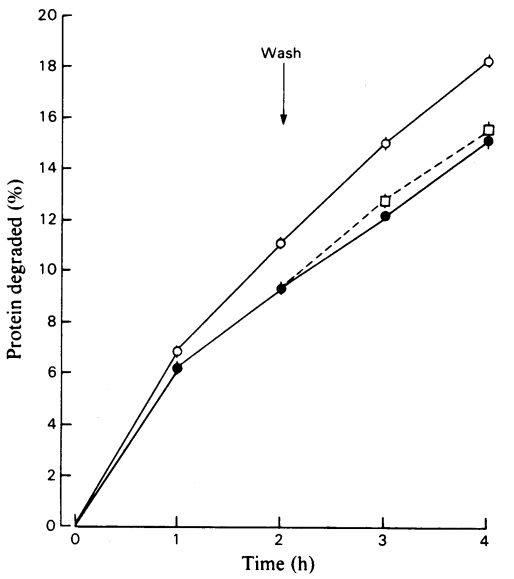

Fig. 4. *Time course of the degradation of endogenous cell protein by yolk sacs*
After a 3 h labelling period and a 90 min chase period, sacs were incubated in 'degradation' medium in the absence (○) or presence (●) of 100 μg of leupeptin/ml of medium. Some sacs were taken at 1 and 2 h for the measurement of protein degraded. The remaining sacs were rinsed three times in leupeptin-free medium and either replaced in similar medium as before or transferred from leupeptin-containing medium to leupeptin-free medium (□) for an additional 1 or 2 h. Each value is the mean ± S.E.M. for seven determinations and is expressed as the cumulative percentage degradation of [^{3}H]leucine-labelled cell protein.

protein also appeared in the medium during incubation of [^{3}H]leucine-labelled yolk sacs. The amount released by 4 h was about 3.5% of the total

tissue radioactivity either under control conditions or when yolk sacs were incubated in the presence of 40μg of leupeptin/ml (Table 2).

Tissue concentrations of adenine nucleotides were measured after 2h incubation of yolk sacs under the same conditions used for the measurement of endogenous protein degradation (Table 2). The ATP content as well as the ATP/ADP and ATP/AMP ratios remained unchanged even in the presence of 100μg of leupeptin/ml.

Effects of various proteinase inhibitors on the degradation of ^{125}I-labelled albumin and endogenous protein

The experiments in Figs. 1 and 2 show that leupeptin is a powerful inhibitor of the lysosomal degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin. Antipain, bestatin, chymostatin, elastatinal and pepstatin were tested to discover whether they too inhibited proteolysis in the yolk-sac system. The effects of leupeptin were again measured to give a more direct comparison of the various inhibitors. This was necessary, as the batch of formaldehyde-denatured ^{125}I-labelled bovine serum albumin differed from that used in the previous experiments. Thus the rate of uptake of the ^{125}I-labelled albumin used here in control experiments was 209 ± 9 ng/mg of yolk-sac protein per h (Table 3), less than observed in the previous control experiments (276 ± 19 ng/mg of yolk-sac protein per h, see Fig. 1).

None of the microbial proteinase inhibitors, present at either 10 or 30μg/ml of incubation medium, modified the rate of uptake of the ^{125}I-

Table 2. *Effects of leupeptin on yolk-sac viability*

Measurements were made on yolk sacs incubated in control medium and in the presence of 50μg of leupeptin/ml for the release experiments and 100μg/ml for the analysis of adenine nucleotides. Values are means ± S.E.M. for the numbers of determinations given in parentheses.

	Control	Leupeptin
Lactate dehydrogenase release (% in 4h)	1.4	1.7
Release of ^{3}H-labelled protein (% in 4h)	3.5 ± 0.2 (10)	3.3 ± 0.2 (8)
Tissue [ATP] (nmol/mg of protein)	18.1 ± 0.8 (5)	17.4 ± 0.9 (5)
Tissue [ADP] (nmol/mg of protein)	3.62 ± 0.16 (5)	3.31 ± 0.27 (5)
Tissue [AMP] (nmol/mg of protein)	1.45 ± 0.07 (5)	1.48 ± 0.11 (5)
Tissue [ATP]/[ADP] ratio	5.06 ± 0.36 (5)	5.39 ± 0.48 (5)
Tissue [ATP]/[AMP] ratio	12.6 ± 0.4 (5)	12.0 ± 0.9 (5)

Table 3. *Effects of microbial proteinase inhibitors on uptake, degradation and accumulation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by yolk sacs*

Uptake and degradation rates are expressed as ng of ^{125}I-labelled albumin/mg of yolk-sac protein per h. The quantity of ^{125}I-labelled albumin remaining associated with the tissue after 3h of incubation is expressed as ng of ^{125}I-labelled albumin/mg of yolk-sac protein. Labelled albumin (1μg/ml) was added 30min after the yolk sacs had been placed in medium containing inhibitor (10 or 30μg/ml) and the sacs were incubated for a further 3h. After an initial lag period of less than 1.0h, the quantity of radioactivity associated with the tissue rose to a steady-state value and remained constant up to 3h, the rate of degradation showed a similar pattern (see Fig. 1). Mean values (±S.E.M.) are reported for the numbers of determinations indicated in parentheses.

Inhibitor (μg/ml)		(1) Rate of uptake (ng/mg of yolk-sac protein per h)	(2) Rate of degradation when steady state is established (ng/mg of yolk-sac protein per h)	(3) Quantity of ^{125}I-labelled albumin associated with tissue after 3h (ng/mg of yolk-sac protein)	(4) Proportion of radioactivity in tissue at 3h that is acid-soluble (%)
None (8)		209 ± 9	205 ± 10	99 ± 6	36.4 ± 1.2
Antipain:	10 (4)	227 ± 9	202 ± 7	150 ± 8	26.3 ± 1.8
	30 (4)	200 ± 13	152 ± 20	219 ± 20	15.7 ± 1.8
Bestatin:	10 (3)	216 ± 18	214 ± 15	108 ± 12	38.4 ± 3.9
	30 (3)	197 ± 18	186 ± 16	107 ± 9	34.7 ± 2.1
Chymostatin:	10 (4)	215 ± 12	181 ± 11	181 ± 15	19.6 ± 2.3
	30 (4)	209 ± 17	174 ± 9	241 ± 15	14.8 ± 2.1
Elastatinal:	10 (3)	200 ± 6	194 ± 2	91 ± 9	36.7 ± 3.1
	30 (3)	196 ± 21	182 ± 18	101 ± 22	30.8 ± 3.5
Leupeptin:	10 (4)	212 ± 34	167 ± 26	200 ± 33	17.7 ± 2.1
	30 (3)	214 ± 24	115 ± 28	314 ± 39	9.6 ± 1.6
Pepstatin:	10 (3)	211 ± 17	207 ± 16	98 ± 10	36.6 ± 2.3
	30 (3)	196 ± 15	196 ± 12	101 ± 13	35.2 ± 2.1

labelled albumin (Table 3, column 1). Leupeptin was found to be the most potent inhibitor of degradation, giving at the higher concentration a rate equal to 53% of the rate of uptake (Table 3, column 2). Antipain and chymostatin also showed substantial dose-dependent inhibition of degradation. Bestatin, elastatinal and pepstatin were ineffective at either 10 or 30 μg/ml.

When an inhibition of ^{125}I-labelled albumin degradation was observed with any of the microbial inhibitors, a corresponding increase was seen in the accumulation of radioactivity by yolk sacs. Thus the quantity of tissue-associated radioactivity increased along the series; control = pepstatin = elastatinal = bestatin $\ll$ antipain = chymostatin $\ll$ leupeptin (Table 3, column 3). The proportion of the tissue-associated radioactivity that was trichloroacetic acid-soluble decreased in the same order (Table 3, column 4), providing evidence that the proteinase inhibitors were indeed acting to prevent tissue degradation of labelled albumin.

Each of the proteinase inhibitors used in the albumin-uptake experiments, and also phosphoramidon, was added to yolk sacs prelabelled with [^{3}H]leucine to determine the extent of inhibition of endogenous protein breakdown. Table 4 shows that leupeptin was more effective than chymostatin or antipain, but none of the other inhibitors altered the rate of protein breakdown over a 4h period. Although not shown in Table 4, the inhibitors were also tested over a 2h incubation period. Neither the order of effectiveness of the proteinase inhibitors nor the percentage inhibition showed any marked differences between the two periods. One further way in which the microbial compounds might inhibit intralysosomal proteolysis of endocytosed proteins is by preventing lysosome–pinosome fusion. This possibility was tested indirectly by determining the effects of the inhibitors on the degradation of ^{125}I-labelled albumin by yolk-sac homogenates. Since the intralysosomal pH within intact yolk sacs is not known, the cell-free extracts were incubated with ^{125}I-labelled albumin at both pH4 and 5.5. Triton X-100 (0.2%) was also included to disrupt lysosomes (Table 5).

At pH4 the order of the effectiveness of the inhibitors in preventing the hydrolysis of ^{125}I-labelled albumin by yolk-sac homogenate was: leupeptin > chymostatin = antipain > bestatin = elastatinal = pepstatin > control. At pH 5.5 the potency of each inhibitor was in general decreased, except for pepstatin, which was more effective at pH 5.5 than at pH 4.

Table 4. *Effects of microbial proteinase inhibitors on the degradation of endogenous yolk-sac proteins*

Values are the percentage of [^{3}H]leucine-labelled protein that is degraded over a 4h incubation period and are expressed as means ± S.E.M., with the numbers of determinations in parentheses. Each inhibitor was dissolved in dimethyl sulphoxide and added to give a concentration of 50 μg/ml.

Inhibitor	Protein degraded (% in 4h)
None	17.42 ± 0.38 (11)
Antipain	15.78 ± 0.62 (10)
Bestatin	17.63 ± 0.29 (10)
Chymostatin	14.80 ± 0.32 (10)
Elastatinal	16.89 ± 0.47 (10)
Leupeptin	14.07 ± 0.42 (8)
Pepstatin	17.38 ± 0.44 (10)
Phosphoramidon	16.98 ± 0.37 (10)

Table 5. *Effects of microbial proteinase inhibitors on the degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by yolk-sac extracts*

Homogenized yolk sacs were incubated in 0.04 M-sodium acetate buffer in the presence of 0.2% Triton X-100 and ^{125}I-labelled albumin with or without each inhibitor (20 μg/ml). Values are means ± S.E.M. for determinations on four extracts.

Inhibitor	Relative rates of degradation (% relative to control rates) at: pH4.0	pH5.5
None	100 ± 3.3	100 ± 8.3
Antipain	14.6 ± 2.5	84.5 ± 3.1
Bestatin	75.0 ± 6.4	98.3 ± 1.0
Chymostatin	11.9 ± 3.7	84.7 ± 9.1
Elastatinal	71.8 ± 7.4	82.5 ± 2.3
Leupeptin	5.3 ± 3.2	49.3 ± 3.8
Pepstatin	77.4 ± 2.7	63.3 ± 8.4

Discussion

Previous studies with rat yolk sacs have shown that the effects of weak bases are not confined to inhibition of intralysosomal proteolysis; concentrations that inhibit proteolysis also decrease pinocytosis as well as the phosphorylation state of adenine nucleotides (Livesey *et al.*, 1980). In contrast with the weak bases, none of the six microbial proteinase inhibitors decreases the rate of pinocytic uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (Fig. 2 and Table 3). Moreover, leupeptin neither modifies the low release rates of endogenous proteins nor alters the tissue concentrations of ATP, ADP or AMP (Table 2). Clearly the microbial proteinase inhibitors do not have the undesirable effects of weak bases.

At the concentration of leupeptin used to test for non-specific effects, the degradation of pinocytosed ^{125}I-labelled albumin is markedly decreased (Figs. 1 and 2), leading to the accumulation of pinocytosed

protein within the tissue. Antipain and chymostatin also inhibit albumin degradation. Inhibition of intralysosomal proteolysis (Fig. 2 and Table 3) does not result in any feedback inhibition of pinocytosis even after prolonged incubation of the yolk sacs. This is consistent with the observation by Roberts *et al.* (1976) that engorgement of the vacuolar system of yolk sacs with non-digestible material does not impair pinocytic capacity. The result contrasts with phagocytosis in guinea-pig macrophages, a process which is inhibited by chymostatin (Nagai *et al.*, 1978).

There is much circumstantial evidence suggesting that antipain, chymostatin and leupeptin inhibit intralysosomal proteolysis in cultured cells (see Knowles & Ballard, 1976; Hopgood *et al.*, 1977; Libby & Goldberg, 1978). The observations made here with ^{125}I-labelled albumin give direct evidence of a lysosomal inhibition, since exogenous albumin is degraded exclusively within the lysosomal system of yolk sacs (Livesey & Williams, 1979). The inhibition of albumin digestion by yolk sacs is greater with leupeptin than with either antipain or chymostatin, whereas pepstatin, elastatinal and bestatin are without detectable effect (Table 3). This pattern of inhibition is reproduced when the breakdown of [^{3}H]leucine-labelled endogenous protein is monitored (Table 4), but less inhibition is observed. A similar pattern is also obtained for the inhibition of ^{125}I-labelled-albumin digestion by cell-free yolk-sac homogenates at pH 4 (Table 5). The last of these observations provides evidence that the effects on endogenous and exogenous protein breakdown in the intact tissue are due to direct inhibition of proteinases rather than an indirect response via an inhibition of the fusion of pinosomes with lysosomes.

Leupeptin and, to a lesser extent, antipain and chymostatin are inhibitors of cathepsin B (Aoyagi & Umezawa, 1975), and leupeptin and chymostatin also inhibit cathepsin L (Kirschke *et al.*, 1977). Hence, either one or both of these cathepsins must be rate-limiting for the degradation of pinocytosed albumin and possibly also for the breakdown of endogenous protein. The smaller degree of inhibition by antipain and chymostatin, compared with leupeptin, observed in each of the three systems (Tables 3, 4 and 5) is in accord with the lower potency of the first two compounds as inhibitors of the cathepsins. These effects on albumin degradation in yolk sacs are in accord with the observation of Huisman *et al.* (1974), who concluded that, for rat liver lysosomes, cathepsin B or some other leupeptin-sensitive enzyme is responsible for the initial, rate-limiting cleavage of peptide bonds in serum albumin.

Pepstatin, a powerful inhibitor of cathepsin D (Umezawa, 1977), did not inhibit the breakdown of either endogenous proteins or pinocytosed albumin. A lack of inhibition by pepstatin in rat yolk sacs was also found by Moore (1975), who used both ^{125}I-labelled albumin and ^{125}I-labelled fibrinogen as substrates. Also pepstatin was a poor inhibitor of ^{125}I-labelled albumin degradation by tissue homogenates at pH 4 (Table 5). One interpretation of these results is that cathepsin D is not rate-limiting for the breakdown of either exogenous or endogenous proteins. The absence of an inhibition of endogenous cell-protein breakdown by pepstatin contrasts with experiments in which liposomally entrapped pepstatin markedly decreased the breakdown of cell proteins in rat liver (Dean, 1975). Likewise, the lack of inhibitory effects of bestatin and elastatinal suggest either that elastase and aminopeptidase (Umezawa, 1977) are absent from yolk-sac lysosomes or that neither is rate-limiting for proteolysis.

An alternative explanation of the lack of inhibition by pepstatin, bestatin or elastatinal in yolk sacs is that these compounds do not attain intralysosomal concentrations sufficient to inhibit the respective proteinases. The lack of a progressively increasing inhibitory effect of leupeptin, and other inhibitory microbial peptides, on the catabolism of denatured albumin (see Fig. 1) suggests that the inhibitors do not continue to accumulate within the tissue, but reach a steady-state intralysosomal concentration consistent with a lower constant rate of albumin catabolism. This would occur if the inhibitors were rapidly inactivated within lysosomes or if newly formed pinosomes fuse only with primary lysosomes. The second of these explanations would imply an extremely rapid turnover of lysosomes, which does not seem feasible, since Wang & Touster (1975) estimated the average half-life of lysosomal constituents to be 4 days. Either explanation is compatible with the rapid recovery of most of the proteolytic activity of the yolk sacs towards ^{125}I-labelled albumin after exposure to leupeptin (Table 1). However, the poor recovery of endogenous protein degradation after exposure to leupeptin can only be explained if the inhibitor enters a pool that is not rapidly depleted after removal of the inhibitor from the incubation medium. A resolution of these findings requires information on the distribution and eventual intracellular fate of the microbial proteinase inhibitors.

Endogenous protein breakdown is far less sensitive to the microbial proteinase inhibitors than is the degradation of pinocytosed albumin (cf. Tables 3 and 4). Moreover, the maximal inhibition of endogenous protein breakdown by leupeptin in hepatoma cells (Knowles & Ballard, 1976), hepatocytes (Hopgood *et al.*, 1977; Seglen *et al.*, 1979) and skeletal muscle incubated *in vitro* (Libby & Goldberg, 1978; Libby *et al.*, 1979) is between 30 and 45%, somewhat greater than the 20% observed in the yolk sacs. The low degree of inhibition of the

endogenous protein breakdown in the yolk sacs suggests either that leupeptin does not reach the catabolic site or that cathepsin B (or the lysosomal system as a whole) does not play a key role in the degradative pathway for intracellular protein. Entry of leupeptin into lysosomes may occur by direct diffusion into the cell followed by autophagocytosis, after which fusion of autophagic vacuoles with lysosomes would deliver both the leupeptin and endogenous proteins to the site of catabolism. Poor diffusion of leupeptin into the cell would account for a low initial inhibition of endogenous proteolysis, but if diffusion was slow a gradually increasing extent of inhibition should occur. Since this does not take place, at least after a 1h lag period (Fig. 4), it is unlikely that inadequate delivery of leupeptin to autophagolysosomes can account for the low inhibition. It is pertinent that weak bases also inhibit endogenous protein breakdown to a much smaller extent than the breakdown of pinocytically captured albumin in yolk sacs (Livesey *et al.*, 1980). Finally, the effects of leupeptin on endogenous protein breakdown in various cell types are not additive with other lysosomotropic agents (Knowles & Ballard, 1976; Hopgood & Ballard, 1980). The most likely explanation of these results is that leupeptin inhibits all intralysosomal proteolysis to the extent seen with denatured albumin (Fig. 2), but only a small proportion of endogenous proteins is degraded within lysosomes. The remaining degradation would occur by some as yet poorly defined pathway in which any lysosomal involvement is not rate-limiting (Knowles & Ballard, 1976; Amenta *et al.*, 1977; Hopgood & Ballard, 1980).

References

Amenta, J. S., Sargus, M. J. & Baccino, F. M. (1977) *Biochem. J.* **168**, 223–227

Aoyagi, T. & Umezawa, H. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B. & Shaw, E., eds.), pp. 429–454, Cold Spring Harbor Laboratory, Cold Spring Harbor

Ballard, F. J. (1971) *Biochem. J.* **121**, 169–178

Ballard, F. J. (1977) *Essays Biochem.* **13**, 1–37

Dean, R. T. (1975) *Nature (London)* **257**, 414–416

Hopgood, M. F. & Ballard, F. J. (1980) *Ciba Found. Symp.: Protein Degradation in Health and Disease*, pp. 205–218, Excerpta Medica, Amsterdam

Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1977) *Biochem. J.* **164**, 399–407

Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1980) *Biochem. J.* **186**, 71–79

Huisman, W., Lanting, L., Doddema, H. J., Bouma, J. M. W. & Gruber, M. (1974) *Biochim. Biophys. Acta* **370**, 297–307

Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S. & Bohley, P. (1977) *Eur. J. Biochem.* **74**, 293–301

Knowles, S. E. & Ballard, F. J. (1976) *Biochem. J.* **156**, 609–617

Kornberg, A. (1955) *Methods Enzymol.* **1**, 441–443

Libby, P. & Goldberg, A. L. (1978) *Science* **199**, 534–536

Libby, P., Ingwall, J. S. & Goldberg, A. L. (1979) *Am. J. Physiol.* **237**, E35–E39

Livesey, G. & Williams, K. E. (1979) *Biochem. J.* **184**, 519–526

Livesey, G., Williams, K. E., Knowles, S. E. & Ballard, F. J. (1980) *Biochem. J.* **188**, 895–903

Moore, A. T. (1975) M.Sc. Thesis, University of Keele

Nagai, K., Nakamura, T. & Koyama, J. (1978) *FEBS Lett.* **92**, 299–302

Roberts, A. V. S., Nicholls, S. E., Griffiths, P. A., Williams, K. E. & Lloyd, J. B. (1976) *Biochem. J.* **160**, 621–629

Seglen, P. O., Grinde, B. & Solheim, A. E. (1979) *Eur. J. Biochem.* **95**, 215–225

Umezawa, H. (1977) *Acta Biol. Med. Germ.* **36**, 1899–1915

Wang, C.-C. & Touster, O. (1975) *J. Biol. Chem.* **250**, 4896–4902