Effects of Weak Bases on the Degradation of Endogenous and Exogenous Proteins by Rat Yolk Sacs

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(Received 19 November 1979)

In rat yolk sacs incubated *in vitro*, the rates of degradation of endogenous [³H]leucine-labelled proteins and of pinocytically ingested ¹²⁵I-labelled bovine serum albumin were both decreased in the presence of either ammonium, methylammonium or ethylammonium ions (0–20 mM) or much lower concentrations of chloroquine (0–500 μM). These effects were also accompanied by an inhibition of pinocytosis, as measured by the rate of uptake of ¹²⁵I-labelled polyvinylpyrrolidone, and by a fall in the [ATP]/[ADP] ratio within the tissue. Re-incubation in inhibitor-free medium of yolk sacs previously exposed to a weak base restored pinocytic and proteolytic capacities, except for tissues exposed to chloroquine at concentrations above 0.1 mM (these appeared to be cytotoxic); an attendant rise in [ATP]/[ADP] ratios to near normal values was also observed. Weak bases, at concentrations that fully arrested the breakdown of ¹²⁵I-labelled albumin, failed to inhibit by more than 45% the degradation of [³H]leucine-labelled endogenous proteins. Since ¹²⁵I-labelled bovine serum albumin has been shown to be degraded entirely intralysosomally by yolk sacs, this suggests either that the hydrolysis of endogenous proteins is shared between lysosomes and some other site or that, unlike ¹²⁵I-labelled albumin, some endogenous proteins can be degraded within lysosomes at abnormally high pH.

Many reports (Wibo & Poole, 1974; Seglen, 1975; Hopgood *et al.*, 1977; Seglen *et al.*, 1979; Poole *et al.*, 1977) indicate that the intracellular degradation of endogenous protein with previously incorporated labelled amino acid can be partially inhibited by addition of a weak base to the medium in which the cells are incubated. Likewise the catabolism of exogenous ¹²⁵I-labelled proteins after their capture by endocytically active mammalian cells (Goldstein *et al.*, 1975; Carpenter & Cohen, 1976; Tolleshaug *et al.*, 1977; Poole *et al.*, 1977) is strongly inhibited under similar conditions. The two sets of observations are compatible with the suggestion that the observed protein breakdown occurs within the vacuolar system, but is inhibited as a consequence of an increase in lysosomal pH produced by the accumulation of weak bases within lysosomes (de Duve *et al.*, 1974; Ohkuma & Poole, 1978). A second effect, inhibition of cathepsin B, has been noted with chloroquine (Wibo & Poole, 1974).

There is some doubt whether more than one-third of the breakdown of intracellular proteins occurs via the lysosomal system in mammalian cells (Knowles & Ballard, 1976; Amenta *et al.*, 1977). On the other hand, denatured ¹²⁵I-labelled bovine serum albumin

is hydrolysed entirely within lysosomes of the rat yolk sac, a tissue with a high rate of pinocytosis (Livesey & Williams, 1979). Accordingly, we have followed the catabolism of both endogenous [³H]leucine-labelled protein and exogenously added ¹²⁵I-labelled albumin in this tissue to determine whether there are selective effects of weak bases on the two processes.

Materials and Methods

Na¹²⁵I (IMS.30), ¹²⁵I-labelled polyvinylpyrrolidone (IM.33P) and L-[³H]leucine (TRK.170) were products of The Radiochemical Centre, Amersham, Bucks., U.K. Medium 199 (TC20) was supplied by Wellcome Reagents, Beckenham, Kent, U.K., and bovine serum albumin (0124t) was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Eagle's minimal essential medium (Eagle, 1959) was freshly prepared with the modifications noted for specific experiments.

Preparation of ¹²⁵I-labelled bovine serum albumin

Bovine serum albumin was iodinated by using a chloramine-T method and subsequently treated with

alkaline formaldehyde in order to denature the protein (Moore *et al.*, 1977).

Yolk-sac incubations

Yolk sacs from 17.5-day-pregnant Wistar rats were dissected and incubated in the absence of serum, as described by Ibbotson & Williams (1979). The temperature was always kept at 37°C and the gas phase was CO₂/O₂ (1:19).

Rates of pinocytosis and of digestion of ¹²⁵I-labelled substrates by yolk sacs

The rates of uptake of ¹²⁵I-labelled albumin or ¹²⁵I-labelled polyvinylpyrrolidone from medium 199 ('uptake' medium) were determined by using either the method of Ibbotson & Williams (1979) or a modified version of this method that enables results to be obtained from a single yolk sac (Livesey & Williams, 1979); rates of capture were expressed as ng of protein/mg of yolk-sac protein per h (Livesey & Williams, 1979). The rate of digestion of an endocytosed ¹²⁵I-labelled protein was expressed both in the same units as uptake and as a percentage of its rate of capture by the yolk-sac tissue. This permitted a quantitative comparison of effects, on intracellular degradation of proteins, of weak bases at concentrations that also modified the rate of pinocytic uptake. The average rate of accumulation of radioactivity, derived from ¹²⁵I-labelled albumin, within the tissue, was expressed as a percentage of the total rate of uptake of the protein by the tissue.

Recovery of capacity to ingest and digest exogenous protein

Yolk sacs were incubated for 1 h in medium containing one of the weak bases. Subsequently the sacs were removed and washed three times, each for 2 min, in 'uptake' medium containing no weak base before being placed in a fresh culture vessel containing 19.0 ml of fresh medium. Uptake and digestion of ¹²⁵I-labelled albumin commenced on addition in 1.0 ml of medium (Livesey & Williams, 1979). Comparable experiments were performed in the continuous presence of weak base.

Measurement of the catabolism of endogenous yolk-sac proteins

Approximately 40 sacs from three or four pregnant animals were transferred to 150 ml of 'labelling' medium [Dulbecco modified Eagle's minimal essential medium (Dulbecco & Freeman, 1959), but with 10 μM-L-leucine and 2 μCi of [³H]leucine/ml] in a 500 ml conical flask and incubated for 3 h with shaking at 90 cycles/min. Subsequently the sacs were rinsed three times in 'degradation' medium {Eagle's minimal essential medium containing 2 mM-L-leucine and 20 mM-N-2-[tris(hydroxymethyl)-methyl]aminoethanesulphonic acid, pH 7.5 (Gunn

et al., 1977)} over a period of 15 min. At this stage amniotic tissue can be readily distinguished from yolk sacs and is discarded. The yolk sacs were transferred to a second 500 ml conical flask containing 150 ml of 'degradation' medium and incubated for 90 min. This 'chase' period was adopted to permit the degradation of unstable proteins.

Protein degradation was measured by transferring single yolk sacs to 20 ml plastic scintillation vials containing 5 ml of 'degradation' medium, together with substances being tested for their effects on protein breakdown, and incubated with shaking, usually for 4 h. At the end of this degradation period 0.5 ml of 100% (w/v) trichloroacetic acid was added and each vial was placed in ice. The vial contents were thoroughly homogenized in a coaxial homogenizer and centrifuged (20000 g·min). Radioactivity in duplicate 100 μl portions of the supernatant was measured with 500 μl of NCS solubilizer and 5 ml of toluene scintillation fluid (Knowles *et al.*, 1975). After the remaining supernatant had been discarded, each pellet was dissolved in 2 ml of 0.5 M-NaOH, and duplicate 50 μl portions were assayed for radioactivity as above.

In addition to the determination of degradation after 4 h, zero-time values were obtained by an equivalent procedure at the beginning of the degradation period. The percentage degradation over the 4 h period was calculated as:

$$\frac{100 \times \text{trichloroacetic acid-soluble radioactivity at 4 h}}{\text{total radioactivity at 4 h}}$$

with a comparable measurement at 0 h subtracted.

Recovery of capacity to degrade endogenous proteins

Yolk sacs, whose rate of intracellular protein breakdown in the presence of a weak base was measured over the period 0–2 h in the above manner, were washed (three times, each for 2 min in 'degradation' medium) and then re-incubated over the period 2–4 h in fresh 'degradation' medium. The percentage degradation in each of these two periods was compared with both that in the continuous presence and that in the complete absence of weak bases.

Measurement of adenine nucleotides in yolk sacs

Yolk sacs were incubated for 3.0 h in 'labelling' medium, but in the absence of L-[³H]leucine, rinsed and re-incubated in 'degradation' medium for a further 90 min as described above. After different intervals, yolk sacs were removed from the incubation medium, quickly blotted and frozen in aluminium tongs that had previously been cooled in liquid N₂. Tissues were stored in liquid N₂ until assayed. The nucleotides in each yolk sac were

extracted by adding 2 ml of 6% (w/v) HClO_4 in 20% (v/v) ethanol, and the tissue was homogenized and centrifuged (10000 g·min) at 0°C. The supernatant was adjusted to pH 6.5 at 0°C with 2.5 M- KHCO_3 , and the precipitated KClO_4 was removed by centrifugation. ATP and ADP in these neutralized extracts were measured as previously described (Ballard, 1971).

The precipitate obtained on adding HClO_4 was dissolved in 2 ml of 0.5 M- NaOH , and the protein content of the tissue was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Release of ^{125}I -labelled polyvinylpyrrolidone, β -N-acetylglucosaminidase and lactate dehydrogenase from yolk sacs

The rate of release of ^{125}I -labelled polyvinylpyrrolidone from yolk sacs that had previously accumulated this substrate was monitored by the method of Williams *et al.* (1975a), and the rates of release of β -N-acetylglucosaminidase (EC 3.2.1.30) and lactate dehydrogenase (EC 1.1.1.27) were measured by the method of Roberts *et al.* (1976). Incubation was performed in medium 199 in the absence or in the presence of one of the weak bases.

Statistical treatment

All values in Tables and Figures are expressed as means $\pm$ S.D., with the numbers of determinations shown in parentheses.

Fig. 1. Time-course of the degradation of ^3H leucine-labelled endogenous protein by rat yolk sacs

The results show the percentage degradation of labelled protein during incubation of yolk sacs in 'degradation' medium in the absence (●) or in the presence (○) of 20 mM- NH_4Cl . Experimental details are given in the text.

Results

Time-course of the degradation of endogenous and exogenous proteins by yolk sacs

On incubating yolk sacs in which endogenous proteins had previously been labelled with ^3H leucine, trichloroacetic acid-insoluble radioactive material was progressively converted to acid-soluble products (Fig. 1). Over the 4 h incubation period, the mean rate of degradation was 3.6%/h, but showed a gradual decrease with time. Fig. 1 also shows the inhibitory effect of 20 mM- NH_4Cl on proteolysis.

The equivalent time-course of proteolysis of an exogenous protein, ^{125}I -labelled albumin, is shown in Fig. 2. In agreement with previous reports (see Livesey & Williams, 1979), after approx. 1 h trichloroacetic acid-soluble hydrolysis products were released into the incubation medium at a constant rate (223 ng/mg of yolk-sac protein per h) and the quantity of radioactivity associated with the tissue

Fig. 2. Time course of the digestion of exogenous ^{125}I -labelled albumin by rat yolk sacs

The results at each time interval were derived from two yolk sacs incubated separately in medium containing ^{125}I -labelled albumin (1 $\mu\text{g}/\text{ml}$) and either (a) no weak base or (b) 20 mM- NH_4Cl . Experimental details are given in the text. The points represent trichloroacetic acid-soluble radioactivity released into the incubation medium (●) and tissue-associated radioactivity (○).

became constant, indicating that pinocytosis was the rate-limiting step in the degradation of this exogenous protein. Fig. 2(b) shows that 20 mM-NH₄Cl completely inhibits the intralysosomal digestion of

¹²⁵I-labelled albumin, but the uptake of this protein remains linear with time, although it occurs at a greatly decreased rate compared with control experiments (Fig. 2a).

Effect of weak bases on pinocytosis

The effect of increasing concentrations of NH₄Cl on the rate of uptake of ¹²⁵I-labelled polyvinylpyrrolidone, a substrate that is captured entirely by fluid-phase pinocytosis (Roberts *et al.*, 1977; Ibbotson & Williams, 1979), is shown in Fig. 3. The rate of uptake of this substrate in the presence of 20 mM-NH₄Cl was 16% of the rate in corresponding controls.

The relative rate of uptake of ¹²⁵I-labelled albumin was, likewise, decreased to 10% in the presence of 20 mM-NH₄Cl. Over the same concentration range, methylammonium chloride and ethylammonium chloride had effects similar to those of NH₄Cl on the uptake of ¹²⁵I-labelled albumin, with uptake inhibited to 42 and 34% respectively at a concentration of 5 mM and to 9 and 10% respectively at 20 mM. Uptake of albumin was more readily inhibited by chloroquine; this compound decreased uptake to 47% at a concentration of 0.1 mM.

Prelabelling of tissue with ¹²⁵I-labelled polyvinylpyrrolidone was used to test for cytolytic effects of the weak bases. Thus, in agreement with Ibbotson &

Fig. 3. Effect of ammonium chloride on the uptake of ¹²⁵I-labelled polyvinylpyrrolidone by yolk sacs

Yolk sacs were incubated in medium containing different concentrations of NH₄Cl for 30 min before the addition of ¹²⁵I-labelled polyvinylpyrrolidone (2 μg/ml). Incubations were continued for 3 h for the measurement of uptake. Experimental details are given in the text.

Fig. 4. Effects of ammonium chloride concentration on rates of uptake and accumulation of ¹²⁵I-labelled albumin by yolk sacs

Yolk sacs were incubated in medium containing different concentrations of NH₄Cl for 30 min before addition of ¹²⁵I-labelled albumin (1 μg/ml). Incubations were continued for 3 h. Experimental details are given in the text. The points represent rate of uptake (●) and total radioactivity associated with the tissue (○).

Williams (1979), control tissue released 1.2% of the tissue-associated radioactivity/h over a 2 h period. Although 0.2 mM-chloroquine caused a 10-fold increase in the rate of release of polyvinylpyrrolidone, the effects of 20 mM-ammonia, -methylamine or -ethylamine were minimal. Furthermore, the release of β -N-acetylglucosaminidase and lactate dehydrogenase that accompanies cell lysis was less than 0.2%/h in all incubations except at chloroquine concentrations above 0.1 mM.

Effects of weak bases on protein breakdown

Once the effects of weak bases on pinocytosis had been established and quantified (Fig. 3), any residual effect on the proteolytic breakdown of exogenous proteins could be examined. Qualitatively, some such effect was evident from the observation that, although the rates of pinocytosis and proteolysis both fell sharply on increasing the concentration of NH_4Cl in the incubation medium, the amount of tissue-associated radioactivity first rose above the value found in tissues from similar experiments in the absence of weak bases, but fell as inhibition of pinocytosis became virtually complete (Fig. 4). Other experiments confirmed that this radioactivity was predominantly acid-insoluble. By expressing the rate of protein breakdown as a percentage of the rate of pinocytosis of ^{125}I -labelled albumin in the presence of a given concentration of a weak base, a quantitative measure of the effect of weak bases on proteolysis alone could be obtained (Fig. 5). Fig. 5 also shows the effect of weak bases on the rate of degradation of endogenous ^3H -leucine-labelled proteins. At all concentrations of NH_4Cl , the extent of inhibition of the digestion of

^{125}I -labelled albumin was greater than that of endogenous proteins; 20 mM- NH_4Cl caused approx. 100% inhibition of the digestion of the exogenous protein but only 45% inhibition of the breakdown of

Fig. 5. Effects of ammonium chloride on the rates of degradation of endogenous ^3H -leucine-labelled protein and pinocytosed ^{125}I -labelled albumin by yolk sacs. Experimental details are given in the text. Values at different concentrations of NH_4Cl show the relative rates of degradation (control = 100%) of endogenous ^3H -leucine-labelled yolk-sac protein (○) or the rates of degradation of exogenous ^{125}I -labelled albumin, expressed as a percentage of its rate of uptake (●).

Table 1. Effects of weak bases on the rate of degradation of endogenous ^3H -leucine-labelled yolk-sac proteins and on the relative rate of digestion of pinocytically captured formaldehyde-denatured ^{125}I -labelled bovine serum albumin. Yolk sacs whose endogenous proteins had been pulse-labelled with L- ^3H -leucine and incubated for 1.5 h to permit degradation of unstable proteins were re-incubated for a further 4 h in either the presence or the absence of a weak base. These results are expressed as percentage inhibitions relative to the corresponding rate of hydrolysis in base-free medium. The rates of degradation of ^{125}I -labelled albumin, captured pinocytically from medium containing a weak base, were also measured. Because the weak bases not only affect the degradation of exogenous protein but also decrease the rate of pinocytic capture, rates of degradation are expressed relative to the rate of uptake of ^{125}I -labelled albumin in the presence of a specific concentration of the weak base.

Inhibitor	Concn. (mM)	Inhibition of proteolysis (%)	
		Endogenous protein	Exogenous protein
Ammonium chloride	5	25.1 ± 6.3 (6)	31.2 ± 4.9 (3)
	20	47.1 ± 5.5 (5)	94.7 ± 7.2 (3)
Methylammonium chloride	5	34.2 ± 7.7 (6)	33.0 ± 13.5 (3)
	20	44.6 ± 7.5 (5)	102.8 ± 9.2 (3)
Ethylammonium chloride	5	27.3 ± 4.7 (4)	41.0 ± 13.8 (3)
	20	45.6 ± 3.9 (4)	95.5 ± 1.4 (3)
Chloroquine phosphate	0.01	0.4 ± 7.5 (4)	9.9 ± 3.3 (3)
	0.10	16.4 ± 7.5 (4)	73.4 ± 6.9 (3)
	0.50	36.1 ± 9.9 (3)	98.8 ± 2.1 (3)

endogenous proteins. Equivalent experiments to those described above, but performed with either the same concentrations of methylammonium and ethylammonium ions or much lower concentrations of chloroquine in place of ammonium ions, gave essentially the same pattern of results (Table 1).

Recoveries of pinocytic and proteolytic activities

After exposure for 1 h to 6.3 mM-NH₄Cl, a concentration that induces 25% inhibition of pinocytosis and 50% inhibition of the degradation of ¹²⁵I-labelled albumin, yolk sacs immediately showed a complete recovery of both pinocytic and proteolytic capacities. Yolk sacs similarly exposed to 20 mM-NH₄Cl, which induces an 80% inhibition of pinocytosis and a 100% inhibition of degradation, subsequently recovered 95–100% of their proteolytic capacity and 84–90% of their pinocytic capacity (Table 2). Both recoveries were maximal within 8 min of washing the tissue, indicating that

ammonia rapidly diffuses out of the tissue on re-incubation in base-free medium.

Similar results for the recovery of proteolytic activity towards [³H]leucine-labelled endogenous proteins after exposure to NH₄Cl (20 mM) are shown in Table 3. After 2 h exposure of tissues to this concentration of NH₄Cl, the rate of degradation of endogenous proteins on subsequent re-incubation for 2 h was markedly higher than the rate observed in the continuous presence of the base and also appeared to be greater than that in the absence of any exposure to weak base. In contrast, after exposure to 500 μM-chloroquine only slight recovery of proteolysis of endogenous proteins was observed (Table 3).

Effects of weak bases on adenine nucleotide concentrations

The mean amount of ATP found in freshly dissected yolk sacs was 16.27 ± 1.33 nmol/mg of

Table 2. *Recovery of pinocytic and proteolytic activities towards ¹²⁵I-labelled albumin in yolk sacs previously exposed to ammonium chloride*

After 1 h exposure to NH₄Cl at the concentrations indicated, yolk sacs were washed, transferred to base-free 'uptake' medium and incubated for 8, 60 or 120 min before addition of ¹²⁵I-labelled albumin (1 μg/ml of medium). Both uptake and degradation were measured. The effects of the continuous presence of NH₄Cl on the uptake and digestion of ¹²⁵I-labelled albumin were also determined. Experimental details are given in the text.

Concn. of NH ₄ Cl (mM)	Continuous presence of NH ₄ Cl in medium		Recovery after 1 h exposure to NH ₄ Cl		
	Rate of uptake (% of control)	Rate of proteolysis (% of rate of uptake)	Period before addition of substrate (min)	Rate of uptake (% of control)	Rate of proteolysis (% of rate of uptake)
6.3	76.1 ± 2.5	54.4 ± 8.8	8	119.4 ± 13.3	99.2 ± 1.2
			60	91.2 ± 13.6	102.2 ± 4.0
			120	98.5 ± 14.7	96.4 ± 2.5
20.0	20.0 ± 8.5	-0.04 ± 1.18	8	89.6 ± 17.8	102.9 ± 2.3
			60	84.2 ± 8.5	98.9 ± 2.4
			120	86.4 ± 14.9	94.9 ± 2.5

Table 3. *Recovery of proteolytic activity towards endogenous [³H]leucine-labelled yolk-sac proteins in tissues previously exposed to weak bases*

The rates of degradation over 2 h intervals were measured in yolk sacs that were either continuously exposed to a weak base or were exposed only from 0 h to 2 h and re-incubated from 2 to 4 h in the absence of base. Experimental details are given in the text. Rates of degradation over the periods indicated were calculated from results obtained with single yolk sacs.

Base	Concn. (mM)	% of endogenous protein degraded over the interval			
		0–2 h		2–4 h	
		Base present	Base absent	Base present	Base absent
None (control)	0	—	8.60 ± 0.96 (9)	—	6.30 ± 0.99 (11)
Ammonium chloride	20	5.56 ± 0.54 (6)	—	4.07 ± 0.76 (6)	7.56 ± 1.11 (7)
Methylammonium chloride	20	4.60 ± 0.80 (6)	—	3.64 ± 1.06 (7)	5.88 ± 0.95 (7)
Chloroquine phosphate	0.5	5.23 ± 0.53 (7)	—	2.88 ± 0.93 (9)	3.38 ± 1.38 (9)

Table 4. Adenine nucleotide contents of yolk sacs either continuously exposed to a weak base or re-incubated in base-free medium after such exposure

Yolk sacs were either exposed continuously to a weak base (5 or 20mM) for 2h or exposed in this manner and then re-incubated for either 1 or 2h in base-free medium before determination of their nucleotide content as described in the text. The values of the ATP content (nmol/mg of yolk-sac protein) are reported, with the corresponding [ATP]/[ADP] ratios, in *italics*, in square brackets.

Base	Concn. (mM)	ATP content of tissue (nmol/mg of yolk-sac protein) and [ATP]/[ADP]		
		Continuous presence of base (2h)	Recovery (1h) in base-free medium	Recovery (2h) in base-free medium
None (control)	0	14.19 ± 1.33 (12) <i>[5.00 ± 0.99]</i>	13.61 ± 1.10 (6) <i>[5.11 ± 0.51]</i>	14.12 ± 1.46 (5) <i>[5.73 ± 0.80]</i>
Ammonium chloride	5	12.75 ± 1.25 (6) <i>[4.69 ± 0.54]</i>	—	—
	20	10.84 ± 1.11 (13) <i>[3.19 ± 0.52]</i>	11.37 ± 0.92 (6) <i>[4.92 ± 0.69]</i>	11.54 ± 0.48 (4) <i>[5.10 ± 0.52]</i>
Methylammonium chloride	5	12.09 ± 0.87 (6) <i>[4.28 ± 0.34]</i>	—	—
	20	9.80 ± 1.07 (13) <i>[2.76 ± 0.42]</i>	10.07 ± 1.21 (6) <i>[4.70 ± 0.78]</i>	9.83 ± 1.26 (5) <i>[4.50 ± 0.24]</i>
Ethylammonium chloride	5	13.74 ± 0.13 (3) <i>[5.41 ± 0.47]</i>	—	—
	20	11.68 ± 0.91 (3) <i>[3.65 ± 0.34]</i>	—	—

yolk-sac protein and the corresponding mean [ATP]/[ADP] ratio was 5.32 ± 0.92 . Incubation in 'degradation' medium for up to 8.5h caused a slight decrease in the mean ATP content, but there was no attendant change in the [ATP]/[ADP] ratio in the tissue (Table 4).

The effects of the weak bases other than chloroquine, which interferes with the nucleotide assay, on these parameters are shown in Table 4. Incubation in the presence of either of the three weak bases decreased, in a dose-dependent manner, both the concentration of ATP in the tissue and the [ATP]/[ADP] ratio (Table 4). After a 2h exposure to NH_4Cl or methylammonium chloride (20mM) there was a significant rise in the [ATP]/[ADP] ratio on re-incubating tissues for either 1 or 2h in base-free medium, but no substantial change in the ATP content was observed (Table 4).

Discussion

The endogenous [^3H]leucine-labelled proteins of yolk-sac tissue are broken down at a gradually decreasing rate over a 4h interval; in the presence of 20mM- NH_4Cl this rate is decreased by approx. 45% (Fig. 1). Similar results were obtained when Reuber H35 hepatoma cells (Knowles & Ballard, 1976) or isolated hepatocytes (Hopgood *et al.*, 1977) were incubated in the absence and in the presence of 10mM- NH_4Cl . In contrast, corresponding experiments show the degradation of an exogenous protein, formaldehyde-denatured ^{125}I -labelled bovine

serum albumin, to be almost completely inhibited by 20mM- NH_4Cl (Fig. 2). In hepatocytes, NH_4Cl was slightly less effective than its analogues as an inhibitor of proteolysis (Hopgood *et al.*, 1977), but no such difference was observed in yolk sacs (Table 1), presumably because the tissue is unable to eliminate ammonia by converting it into urea.

In addition to inhibiting intralysosomal proteolysis, ammonium ions also strongly inhibit the pinocytic capture of ^{125}I -labelled albumin (Fig. 4). Since the rate of capture of ^{125}I -labelled polyvinylpyrrolidone, a marker of fluid-phase pinocytosis (Williams *et al.*, 1975a; Roberts *et al.*, 1977), is decreased by NH_4Cl in a similar dose-dependent manner to that of ^{125}I -labelled albumin (Figs. 3 and 4), it appears that ammonia inhibits the rate of pinosome formation rather than the adsorption of albumin on yolk-sac cells (Ibbotson & Williams, 1979). Inhibition of pinosome formation offers a satisfactory explanation of the biphasic nature of the plot of radioactivity (derived from ^{125}I -labelled albumin) associated with the yolk-sac tissue against NH_4Cl concentration (Fig. 4). Thus the amount of acid-insoluble radioactivity accumulated by the tissue rises with increasing NH_4Cl concentration as proteolysis is inhibited, but then falls as inhibition of pinocytosis becomes the predominant effect.

Ammonium ions could inhibit both proteolysis and pinocytic capture simply by being cytotoxic, but this explanation is unlikely. Yolk sacs whose lysosomal system had been loaded *in vitro* with ^{125}I -labelled polyvinylpyrrolidone leaked little of this

marker on re-incubation in medium containing 20 mM-NH₄Cl. Likewise, only very small amounts of lactate dehydrogenase and β -N-acetylglucosaminidase were released by the tissue in the presence of high concentrations of NH₄Cl. Furthermore, within 8 min, tissues previously exposed to 20 mM-NH₄Cl fully recovered their pinocytic capacity (Table 2) and their proteolytic capacity towards exogenous (Table 2) and endogenous (Table 3) proteins. However, recovery of proteolytic capacity towards endogenous proteins after exposure to 0.5 mM-chloroquine was poor (Table 3). Perhaps chloroquine diffuses out of lysosomes slowly compared with ammonia since, 24 h after exposure to ¹⁴C-labelled chloroquine, mouse macrophages still retained 10–15% of the radioactivity initially associated with the cells (Fedorko *et al.*, 1968). A similar difference in the restoration of lysosomal acid pH in macrophages previously exposed to ammonia and chloroquine has been reported by Ohkuma & Poole (1978). But concentrations of chloroquine in excess of 0.1 mM are definitely cytotoxic to yolk-sac cells, causing high rates of loss of ¹²⁵I-labelled polyvinylpyrrolidone and endogenous enzymes from the tissue.

Certain metabolic inhibitors when present in the incubation medium readily inhibit pinocytosis in the rat yolk sac (Duncan & Lloyd, 1978; Livesey & Williams, 1979), giving indirect evidence that this process is energy-dependent. The degree of pinocytic activity in yolk sacs either exposed continuously to NH₄Cl or its analogues or recovering in base-free medium correlates well with the ATP concentration and even more closely with the [ATP]/[ADP] ratio in the tissue (Table 4). Thus NH₄Cl and its analogues have effects that are not confined to the lysosomal system and, by interfering with the energy status of the cells, could inhibit intracellular proteolysis at any stage that is energy-dependent.

Since weak bases lower the rate of pinocytosis in yolk sacs and since this process is rate-limiting in the overall process of ingestion and proteolysis of ¹²⁵I-labelled albumin (Williams *et al.*, 1975b; Moore *et al.*, 1977), it was essential to normalize the rates of uptake of the exogenous protein before assessment of the effects of different concentrations of a weak base on intralysosomal proteolysis. When this was done (Table 1), a given concentration of a weak base still showed a much greater effect on the degradation of the exogenous protein than on the degradation of endogenous protein, indicating that inhibition of the rate of pinocytosis of the exogenous protein cannot by itself explain the observed differences in the rates of proteolysis. It seems likely that at least some endogenous proteins are degraded within the lysosomal system after capture by either autophagy or lysosomal involution (Dean, 1977).

These uptake processes too may be rate-limiting and energy-dependent, so that the observed rates of proteolysis of endogenous protein in the presence of weak bases would need to be normalized in a manner equivalent to that applied to the rates of digestion of exogenous proteins. The necessary factors cannot be calculated, but they would only serve to accentuate further the differences between the rates of degradation of endogenous and exogenous proteins over those reported in Table 1 and Fig. 5.

Inhibition of digestion of endogenous and exogenous proteins could possibly result from weak bases inhibiting fusion of autophagosomes and heterophagosomes with lysosomes. However, this explanation seems unlikely, since ¹²⁵I-labelled insulin B-chain, like ¹²⁵I-labelled albumin, appears to be digested within lysosomes (G. Livesey & K. E. Williams, unpublished work), but the only effect of 20 mM-NH₄Cl is to decrease the rate of pinocytic capture of this polypeptide. This finding further suggests that, like insulin B-chain, certain exogenous proteins may still be readily degraded in lysosomes even at an abnormally high pH value sufficient to inhibit fully the degradation of ¹²⁵I-labelled albumin. Thus intralysosomal concentrations of weak bases sufficient to make lysosomal-enzyme activities rate-limiting in the hydrolysis of ¹²⁵I-labelled albumin may be inadequate to produce the same effect with endogenous proteins. But Poole *et al.* (1977) have investigated the inhibitory effects of weak bases on the degradation of labelled, exogenously added, macrophage protein by macrophages and reported inhibitions of 60% with 10 mM-NH₄Cl and 68% with 0.1 mM-chloroquine. The degradation of endogenous macrophage protein was not affected under the same conditions, indicating that the differential effect with macrophages is even more extreme than that shown in the yolk-sac experiments and cannot readily be attributed to differences in susceptibility of individual proteins to attack by lysosomal enzymes.

We conclude that, although ammonia is an effective inhibitor of intracellular protein degradation and has minimal cytotoxic effects, its action is not limited to an increase in lysosomal pH, since pinocytosis is markedly inhibited and the energy status of the cells is considerably altered. In spite of this complication in comparing the degradation of exogenous and endogenous proteins, it appears most likely that a substantial fraction of endogenous protein breakdown occurs via a non-lysosomal pathway.

We thank Keele University for support of this work by a Research Studentship awarded to G. L.

References

- Amenta, J. S., Sargus, M. J. & Baccino, F. M. (1977) *Biochem. J.* **168**, 223–227
- Ballard, F. J. (1971) *Biochem. J.* **121**, 169–178
- Carpenter, G. & Cohen, S. (1976) *J. Cell Biol.* **71**, 159–171
- Dean, R. T. (1977) *Biochem. J.* **168**, 603–605
- de Duve, C., de Barsey, T., Poole, B., Trouet, A., Tulkens, P. & van Hoof, F. (1974) *Biochem. Pharmacol.* **23**, 2495–2531
- Dulbecco, R. & Freeman, G. (1959) *Virology* **8**, 396–397
- Duncan, R. & Lloyd, J. B. (1978) *Biochim. Biophys. Acta* **544**, 647–655
- Eagle, H. (1959) *Science* **130**, 432–437
- Fedorko, M. E., Hirsch, J. G. & Cohn, Z. A. (1968) *J. Cell Biol.* **38**, 377–391
- Goldstein, J. L., Brunschede, G. Y. & Brown, M. S. (1975) *J. Biol. Chem.* **250**, 7854–7862
- Gunn, J. M., Clark, M. G., Knowles, S. E., Hopgood, M. F. & Ballard, F. J. (1977) *Nature (London)* **266**, 58–60
- Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1977) *Biochem. J.* **164**, 399–407
- Ibbotson, G. E. & Williams, K. E. (1979) *Biochem. J.* **178**, 785–792
- Knowles, S. E. & Ballard, F. J. (1976) *Biochem. J.* **156**, 609–617
- Knowles, S. E., Gunn, J. M., Hanson, R. W. & Ballard, F. J. (1975) *Biochem. J.* **146**, 595–600
- Livesey, G. & Williams, K. E. (1979) *Biochem. J.* **184**, 519–526
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Moore, A. T., Williams, K. E. & Lloyd, J. B. (1977) *Biochem. J.* **164**, 607–616
- Ohkuma, S. & Poole, B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3327–3331
- Poole, B., Ohkuma, S. & Warburton, M. J. (1977) *Acta Biol. Med. Germ.* **36**, 1777–1788
- Roberts, A. V. S., Nicholls, S. E., Griffiths, P. A., Williams, K. E. & Lloyd, J. B. (1976) *Biochem. J.* **160**, 621–629
- Roberts, A. V. S., Williams, K. E. & Lloyd, J. B. (1977) *Biochem. J.* **168**, 239–244
- Seglen, P. O. (1975) *Biochem. Biophys. Res. Commun.* **66**, 44–52
- Seglen, P. O., Grinde, B. & Solheim, A. E. (1979) *Eur. J. Biochem.* **95**, 215–225
- Tolleshaug, H., Berg, T., Nilsson, M. & Norum, K. R. (1977) *Biochim. Biophys. Acta* **499**, 73–84
- Wibo, M. & Poole, B. (1974) *J. Cell Biol.* **63**, 430–440
- Williams, K. E., Kidston, E. M., Beck, F. & Lloyd, J. B. (1975a) *J. Cell Biol.* **64**, 113–122
- Williams, K. E., Kidston, E. M., Beck, F. & Lloyd, J. B. (1975b) *J. Cell Biol.* **64**, 123–134