Effects of inhibitors on aldolase breakdown after its microinjection into HeLa cells

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1. The regulation of protein breakdown as well as the generation of intermediates in the pathway from intact protein to amino acids was investigated by using ^3H -labelled *N*-ethylmaleimide-modified aldolase (NEM-aldolase) as an indicator protein after its microinjection into HeLa cells. 2. NEM-aldolase degradation to acid-soluble products proceeded at a slower rate than that of endogenously labelled total cell protein, and was inhibited to a greater extent by 3-methyladenine, leupeptin and NH_4Cl . The combination of leupeptin plus NH_4Cl was particularly effective, decreasing the NEM-aldolase breakdown rate by 90 %. 3. Measurements of the loss of radioactivity from the aldolase band located from fluorograms after SDS/polyacrylamide-gel electrophoresis showed that NEM-aldolase breakdown was much more rapid when measured by this method. The effects of insulin, 3-methyladenine, leupeptin and NH_4Cl on this breakdown were also substantial. 4. Substantial amounts of peptide intermediates in the breakdown pathway of NEM-aldolase accumulated in cells. The production of small intermediates (less than 30 kDa) accounted for approx. 40 % of the NEM-aldolase degraded in control cultures. Addition of NH_4Cl increased the proportion of these intermediates. Large intermediates, between 31 and 38 kDa, were particularly evident in the presence of the cysteine proteinase inhibitor leupeptin, but almost no small intermediates were detected. 5. The results are best explained by the degradation of NEM-aldolase being predominantly a lysosomal process, with cysteine proteinases involved in early proteolytic steps and other proteinases that have acid pH optima required for the complete catabolism of small intermediates.

INTRODUCTION

Studies on the regulation of intracellular protein breakdown have shown that a substantial proportion of total catabolism occurs via a pathway that can be inhibited by lysosomotropic agents or by agents that prevent the autophagic process (Knowles & Ballard, 1976; Ballard, 1987; Seglen, 1987). It has been inferred from such inhibitor investigations, as well as from electron-microscope analysis and cell-organelle-isolation experiments (Seglen, 1987; Mortimore, 1987; Pfeifer, 1987), that both membrane and soluble proteins are engulfed within autophagic vacuoles and subsequently degraded after fusion of the vacuoles with lysosomes. Regulation occurs because the rate of engulfment is controlled by hormones and nutrients (Seglen, 1987; Mortimore, 1987; Pfeifer, 1987). A second degradative pathway must occur, because the breakdown of a proportion of the total cell protein is not susceptible to inhibitors of the autophagic lysosomal system (Ballard, 1987; Seglen, 1987). This pathway seems to be selective towards more labile proteins (Knowles & Ballard, 1976), and includes, but is apparently not limited to, a series of reactions that involve ubiquitin (Ciechanover *et al.*, 1984; Parag *et al.*, 1987; Carlson *et al.*, 1987).

Notwithstanding the considerable information on the regulation of protein breakdown, very little is known about intermediate steps in the overall pathways involved. The use of antibodies to detect breakdown products requires prior purification of the products from

large amounts of tissue (Reznick *et al.*, 1985; Toda & Ohashi, 1986), whereas relatively specific labelling methods, such as biotin incorporation (Chandler & Ballard, 1986, 1988), have the disadvantage that the detected products could be derived from more than one protein. Microinjection of labelled pure proteins into cells offers a promising approach. Unfortunately, problems with the purity of the injected protein and its low rate of transfer have limited progress in this area. Recently we noted that modification of aldolase with *N*-ethylmaleimide (NEM) produced up to a 20-fold increase in microinjection, but the half-life of the transferred enzyme was not changed (Hopgood *et al.*, 1988). Here we have used NEM-modified aldolase to examine the breakdown products generated as well as to investigate the regulatory processes involved in the catabolic pathway.

MATERIALS AND METHODS

Materials

L-[4,5- ^3H]Leucine (64 Ci/mmol) was obtained from Amersham, Surry Hills, N.S.W., Australia; NEM was from Sigma Chemical Co., St. Louis, MO, U.S.A., and 3-methyladenine was from Fluka A.G., Buchs, Switzerland. The sources of other chemicals and materials were as described previously (Knowles *et al.*, 1988; Hopgood *et al.*, 1988).

Labelling and inactivation of aldolase

Aldolase was labelled with ^3H and stored in liquid

Abbreviation used: NEM, *N*-ethylmaleimide.

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N₂ as described previously (Hopgood *et al.*, 1988). Thiol modification of the labelled enzyme was performed by addition of NEM (Kowal *et al.*, 1965) for 30 min at 25 °C (Hopgood *et al.*, 1988). The single batch of NEM-aldolase used in the present investigation retained 8 % of its initial catalytic activity after this treatment. Once the inactivation process had been completed, the NEM-aldolase was loaded into erythrocytes within 1 h to minimize further changes in activity.

Loading of aldolase or NEM-aldolase into erythrocyte ghosts, followed by fusion with HeLa cells

The enzyme was loaded into erythrocyte ghosts by using the pre-swell technique (Rechsteiner, 1982) with minor modifications (Knowles *et al.*, 1988). EGTA (1 mM) was present in all loading and washing solutions to inhibit the breakdown of aldolase by Ca²⁺-activated proteinase (M. F. Hopgood, S. E. Knowles & F. J. Ballard, unpublished work).

HeLa cells were harvested, fused to loaded ghosts with inactivated Sendai virus and washed as described previously (Knowles *et al.*, 1988). Finally the fused cells were suspended in sterile Dulbecco's modified Eagle's Minimal Essential Medium containing 10 % (v/v) fetal-bovine serum plus antibiotics and dispensed into 12-place multi-well dishes. Cells were allowed 6 h to attach, after which they were washed with sterile iso-osmotic and hypo-osmotic phosphate buffers (Knowles *et al.*, 1988) and the degradation medium was added.

Measurement of the breakdown rate of microinjected protein

After the re-attachment period the cell monolayers were incubated for 9, 18, 27 and 36 h in 1 ml of Dulbecco's medium plus 1 % fetal-bovine serum and test compounds. The potential regulators being tested were added to give the following concentrations: 20 mM-NH₄Cl, 1 µM-insulin, 10 mM-3-methyladenine and 100 µg of leupeptin/ml. At the end of the incubation period, medium and cell monolayer were harvested as trichloroacetic acid-soluble and -insoluble fractions as previously described (Knowles *et al.*, 1988). The acid-insoluble fractions of cell monolayers were subjected to 10–25 % (w/v)-gradient-polyacrylamide-gel electrophoresis, and the separated peptides were detected by fluorography and quantified by liquid-scintillation counting (Chandler & Ballard, 1985; Hopgood *et al.*, 1988).

The radioactivity in all wash fractions was also measured so that corrections could be made to radioactivity in each gel band to account for differences in plating consistency, harvesting efficiency and recoveries from the gels (Hopgood *et al.*, 1988).

Overall breakdown of microinjected protein was calculated as the percent of the total radioactivity per well that appears as trichloroacetic acid-soluble material.

Measurement of endogenous protein breakdown

HeLa cells were prelabelled with [³H]leucine for 18 h in leucine-free Dulbecco's medium containing 5 % fetal-bovine serum to label all cell proteins. The cells were subsequently suspended by trypsin treatment, fused and plated into 12-place multiwell dishes by using a protocol identical with that described above except that the labelled aldolase in the ghosts was replaced with unlabelled aldolase at the same concentration. After

the 6 h attachment period each monolayer was washed as above and incubated in 1 ml of Dulbecco's medium containing 1 % fetal-bovine serum and the indicated concentrations of other agents. The extent of protein breakdown was calculated from the percentage of labelled protein degraded to acid-soluble products (Ballard *et al.*, 1980), and the effects of specific agents are expressed as percentage changes from the control rate.

RESULTS AND DISCUSSION

Regulation of total protein breakdown

Degradation of leucine-labelled total protein in HeLa cells occurs at approx. 0.5 %/h when the cells are incubated in medium containing 1 % fetal-bovine serum (Table 1). This rate was inhibited by leupeptin, and to a greater extent by NH₄Cl, and co-addition of the two lysosomotropic agents increased the extent of inhibition to 31 % at 18 h or 33 % at 36 h. Inhibitory effects of insulin or 3-methyladenine over 36 h were approximately equal and partially additive, even though both compounds prevent the formation of autophagic vacuoles rather than decrease the catabolism of cellular proteins once they have been engulfed in the vacuoles (Ballard, 1987; Mortimore, 1987; Seglen, 1987). Complete or partial additivity of effects was evident when either inhibitor of the autophagic process was present with leupeptin. However, such a cumulative response was less with NH₄Cl plus either insulin or 3-methyladenine, probably because NH₄Cl by itself was the most effective of the different agents tested. This pattern of responses to a range of protein-breakdown inhibitors is similar to that noted in other cell types and those not subjected to fusion with erythrocyte ghosts (Knowles & Ballard, 1976; Ballard, 1977, 1987; Amenta & Brocher, 1981).

We noted that the inhibition of total cell protein breakdown in the presence of 3-methyladenine, and especially of insulin, increased between the 18 h and 36 h measurement periods (Table 1). Indeed, the control breakdown rate between 18 h and 36 h was 8.79 %, whereas in the presence of insulin the percentage degraded was 6.61 %, giving a 25 % inhibition. Since we had seen this increase in response with time in previous experiments, it seemed likely that either it was a consequence of the fusion process itself or it resulted from transfer of NEM-aldolase to the HeLa cells. The effect was not found when HeLa cells were subcultured without fusion (Table 2, lines 3 and 4). However, when cells were fused with ghosts loaded with either NEM-aldolase or vehicle (5 mM-sodium phosphate, pH 7.4), the breakdown rates measured over the first 18 h were substantially lower than in the control monolayers (Table 2, lines 5 and 7 compared with line 1). Since the rates in the presence of insulin were all similar, a much smaller response to insulin occurred in both fusion treatments. By 36 h the effects of insulin had increased to be nearly equal in the four treatment groups (Table 2), and in all subsequent experiments measurements were taken over longer periods than 18 h. A likely explanation for the initially lower breakdown rate and concomitant poor insulin response in cells subjected to the fusion process is a disruption in cellular homeostatic mechanisms. Thus many toxic agents or conditions lead to a decrease in the rate of protein breakdown (Knowles & Ballard, 1976; Hopgood *et al.*, 1977).

Table 1. Degradation of [³H]leucine-labelled proteins in HeLa cells

Cells were fused with erythrocyte ghosts that had been loaded with non-radioactive NEM-aldolase, then plated and washed, and the degradation was measured over an additional 18 h or 36 h. Values are means $\pm$ S.E.M. for determinations on three wells. Numbers in parentheses represent the percentage inhibition relative to control incubations.

Treatment	Protein degraded (%) over:	
	18 h	36 h
None	8.97 $\pm$ 0.10	17.76 $\pm$ 0.10
Insulin (1 μ M)	8.23 $\pm$ 0.07 (8)	14.84 $\pm$ 0.15 (16)
3-Methyladenine (10 mM)	7.83 $\pm$ 0.01 (13)	14.38 $\pm$ 0.04 (19)
Leupeptin (100 μ g/ml)	7.83 $\pm$ 0.16 (13)	14.96 $\pm$ 0.25 (16)
NH ₄ Cl (20 mM)	6.90 $\pm$ 0.05 (23)	13.19 $\pm$ 0.04 (26)
Insulin, 3-methyladenine	7.27 $\pm$ 0.18 (19)	13.36 $\pm$ 0.02 (25)
Insulin, leupeptin	7.08 $\pm$ 0.10 (21)	12.66 $\pm$ 0.16 (29)
Insulin, NH ₄ Cl	6.74 $\pm$ 0.12 (25)	11.90 $\pm$ 0.07 (33)
3-Methyladenine, leupeptin	7.17 $\pm$ 0.09 (20)	13.06 $\pm$ 0.09 (26)
Leupeptin, NH ₄ Cl	6.21 $\pm$ 0.06 (31)	11.93 $\pm$ 0.04 (33)
Insulin, leupeptin, NH ₄ Cl	6.48 $\pm$ 0.07 (28)	11.17 $\pm$ 0.17 (37)

Table 2. Effect of the fusion process on insulin action in HeLa cells

Cells were prelabelled with [³H]leucine either in 12-place dishes or in bottles. The latter cells were removed with trypsin and either replaced directly into 12-plate dishes (passaged, replated) or fused with erythrocyte ghosts before replating. The ghosts used for fusion were either loaded with 5 mM-sodium phosphate, pH 7.4 (passaged, fused, replated) or this solution containing 1 mg of NEM-aldolase/ml (passaged, fused-injected, replated). When present, insulin was added at 1 μ M. Other details were as described in the Materials and methods section. Values are means $\pm$ S.E.M. for determinations on three wells. Numbers in parentheses represent the percentage inhibition by insulin.

Treatment of cells	Insulin	Protein degraded (%) over:	
		18 h	36 h
None	—	18.05 $\pm$ 0.45	24.36 $\pm$ 0.16
	+	13.78 $\pm$ 0.23 (24)	19.62 $\pm$ 0.14 (20)
Passaged, replated	—	18.10 $\pm$ 0.14	25.53 $\pm$ 0.19
	+	12.60 $\pm$ 0.11 (30)	19.99 $\pm$ 0.04 (21)
Passaged, fused, replated	—	14.83 $\pm$ 0.24	22.81 $\pm$ 0.27
	+	13.32 $\pm$ 0.13 (10)	18.39 $\pm$ 0.15 (19)
Passaged, fused-injected, replated	—	14.82 $\pm$ 0.25	21.40 $\pm$ 0.30
	+	14.01 $\pm$ 0.58 (6)	18.00 $\pm$ 0.21 (16)

Regulation of NEM-aldolase breakdown

The degradation of NEM-aldolase microinjected into HeLa cells occurs at a rate of 12.6%/36 h when calculated from the generation of trichloroacetic acid-soluble products (Table 3). This rate was inhibited by 18% in the presence of insulin, but to much greater extents by leupeptin (52%), NH₄Cl (55%) and especially 3-methyladenine (76%). Co-addition of insulin with the other agents did not increase their effects. However, further inhibition could be demonstrated with combinations of leupeptin and 3-methyladenine or leupeptin and NH₄Cl (Table 3).

Once again, insulin inhibition increased with time, the calculated value being an average of 16% over 27 h and 20% after 36 h. This increase was also observed when insulin was added with other agents, but was of little significance when the total inhibition was 50–90%. The effect of 3-methyladenine on the degradation of NEM-aldolase did not vary with time after microinjection.

In a previous report (Hopgood *et al.*, 1988) we noted that microinjection of NEM-aldolase led to the production of protein fragments, initially in the erythrocyte ghosts and subsequently in the recipient HeLa cells. The first of these degradative processes, the generation of a 38 kDa protein, was substantially decreased in the present investigation by the inclusion of 1 mM-EGTA in all solutions before fusion. However, other protein fragments of lower molecular mass were continually produced throughout the protein-breakdown measurement period (Hopgood *et al.*, 1988). For this reason it is necessary to measure the radioactivity in the aldolase band, in order to quantify the actual loss of aldolase radioactivity. Examples of decay curves for microinjected NEM-aldolase are shown in Fig. 1. Each follows approximately first-order kinetics, thus allowing the calculation of the degradation rate constants that are listed in Table 4 together with the effects of added inhibitors. The breakdown of NEM-aldolase in control cultures pro-

Table 3. Degradation of injected NEM-aldolase to trichloroacetic acid-soluble products

The degradation of NEM-aldolase radioactivity to trichloroacetic acid-soluble products as described in the Materials and methods section was measured. Two values obtained after a 36 h measurement period were combined with two values obtained after a 27 h period, with the latter adjusted by a factor of 36/27. Values quoted are the means $\pm$ S.E.M. for these four determinations over '36 h'. Numbers in parentheses represent the percentage inhibition relative to control cultures.

Treatment	Protein degraded (%)
None	12.56 $\pm$ 0.30
Insulin (1 μ M)	10.32 $\pm$ 0.74 (18)
3-Methyladenine (10 mM)	3.00 $\pm$ 0.12 (76)
Leupeptin (100 μ g/ml)	6.05 $\pm$ 0.21 (52)
NH ₄ Cl (20 mM)	5.65 $\pm$ 0.15 (55)
Insulin, 3-methyladenine	2.87 $\pm$ 0.12 (77)
Insulin, leupeptin	6.41 $\pm$ 0.40 (49)
Insulin, NH ₄ Cl	5.27 $\pm$ 0.42 (58)
3-Methyladenine, leupeptin	1.53 $\pm$ 0.12 (88)
Leupeptin, NH ₄ Cl	1.13 $\pm$ 0.08 (91)
Insulin, leupeptin, NH ₄ Cl	2.11 $\pm$ 0.19 (83)

ceeded with a K_d of 0.384 days⁻¹, and was inhibited moderately by insulin and substantially by leupeptin, NH₄Cl and 3-methyladenine (Table 4). Partial additive effects were found with 3-methyladenine plus leupeptin and leupeptin plus NH₄Cl.

Agents that were effective at decreasing NEM-aldolase breakdown in the present study have produced variable

Table 4. Degradation rate constants for the aldolase-monomer band of injected NEM-aldolase

The degradation rate constants (days⁻¹), $\pm$ S.E.M., were measured from four values at 0 h, two at 9 h, two at 18 h, two at 27 h and two at 36 h of a degradation period. The agents tested were added to HeLa cells at the concentrations indicated. Cell proteins were separated to identify and quantify the aldolase monomer as described in the Materials and methods section. Numbers in parentheses represent the percentage inhibition of breakdown relative to control cultures.

Treatment	Degradation rate constant, K_d (days ⁻¹)
None	0.384 $\pm$ 0.029
Insulin (1 μ M)	0.278 $\pm$ 0.014 (28)
3-Methyladenine (10 mM)	0.149 $\pm$ 0.017 (61)
Leupeptin (100 μ g/ml)	0.180 $\pm$ 0.014 (53)
NH ₄ Cl (20 mM)	0.214 $\pm$ 0.026 (44)
Insulin, 3-methyladenine	0.173 $\pm$ 0.024 (55)
Insulin, leupeptin	0.218 $\pm$ 0.024 (43)
Insulin, NH ₄ Cl	0.264 $\pm$ 0.024 (31)
3-Methyladenine, leupeptin	0.113 $\pm$ 0.012 (71)
Leupeptin, NH ₄ Cl	0.110 $\pm$ 0.014 (71)
Insulin, leupeptin, NH ₄ Cl	0.127 $\pm$ 0.019 (67)

responses in investigations with other microinjected proteins. Thus bovine serum albumin catabolism was not affected by NH₄Cl or leupeptin (Rote & Rechsteiner, 1983; Katznelson & Kulka, 1983), consistent with other evidence that this protein is degraded by a non-lysosomal pathway (Bigelow *et al.*, 1981; McElligott & Dice, 1983). Similar results were found with horseradish peroxidase (Knowles *et al.*, 1988). On the other hand, the breakdown of lactate dehydrogenase, pyruvate kinase, haemoglobin, glutamine synthetase, RNAase A and total cell protein microinjected into several cell types was partially inhibited by lysosomotropic agents or treatments such as high serum concentrations that can be expected to inhibit the formation of autophagic vacuoles (Freikopf-Cassel & Kulka, 1981; Rote & Rechsteiner, 1983; Backer *et al.*, 1983; Slot *et al.*, 1986; Mayer *et al.*, 1986).

One explanation for the differential effects of lysosomotropic agents and autophagy inhibitors on the breakdown of individual microinjected proteins is that the more-labile proteins are degraded predominantly by non-lysosomal mechanisms and the more-stable proteins via autophagy. This pattern applies to proteins endogenously labelled with radioactive amino acids (Knowles & Ballard, 1976; Seglen, 1987) and to the five biotin-containing proteins investigated in 3T3-L1 cells (Chandler & Ballard, 1988). In this context, both bovine serum albumin and horseradish peroxidase, amongst the microinjected proteins studied, have short half-lives (Bigelow *et al.*, 1981; Knowles *et al.*, 1988), whereas the quite large inhibitory effects found in the present investigation are with a relatively stable protein.

Intermediates in the breakdown of NEM-aldolase

A comparison between the results presented in Tables 3 and 4 indicates two anomalies. Firstly, the degradation rate constant calculated from the breakdown of NEM-aldolase to trichloroacetic acid-soluble products in

Fig. 1. Loss of radioactivity from the aldolase monomer after microinjection of NEM-aldolase

HeLa cells were fused, plated, washed and incubated for up to 36 h more before radioactivity was measured in the aldolase monomer after SDS/polyacrylamide-gel electrophoresis of protein samples. Values shown are for control incubations (■), those in the presence of 1 μ M-insulin (●) and those in the presence of 10 mM-3-methyladenine (▲). Means $\pm$ S.E.M. are indicated for the control cultures.

Fig. 2. Relationship between inhibition of NEM-aldolase total breakdown to trichloroacetic acid-soluble products and inhibition of the breakdown of the aldolase monomer

The plotted points represent the mean percentage inhibitions for the ten treatments described in Tables 3 and 4. The dashed line is drawn on the assumption that percentage inhibitions determined by the two methods for the same treatment are equal.

control wells is 0.090 days^{-1} , much slower than the 0.384 days^{-1} found with calculations based on loss of radioactivity in the aldolase monomer. Secondly, for most inhibitors tested the effects were greater on breakdown to trichloroacetic acid-soluble products. This latter difference is clearly evident in Fig. 2, where all the points above the diagonal line fit that pattern. These two results imply that intermediates in the breakdown of NEM-aldolase must have accumulated throughout the degradation measurement period. We considered that the determination of these intermediates could provide useful information on the pathways responsible for the breakdown of NEM-aldolase.

Longer exposure of the fluorograms used to detect the aldolase monomer band for quantification revealed minor bands in cells 6 h after plating (0 h of the measurement period, Fig. 3), as well as the accumulation of some of these bands during the subsequent 36 h. It should be noted that only trichloroacetic acid-insoluble radioactivity was applied to the gels as any acid-soluble, low-molecular-mass breakdown products would have been extracted previously from the cell monolayers. In the presence of 3-methyladenine a band at 36 kDa became more intense, an increase in fluorogram density in bands between approx. 30 kDa and especially at 36 kDa was seen with leupeptin treatment, and the addition of NH_4Cl led to the accumulation of several bands between the buffer front and 20 kDa (Fig. 3). Quantification of the individual bands detected by fluorography was carried out for all lanes, and the values were combined as large intermediates (approx. 31–38 kDa) and small intermediates (buffer front to 30 kDa). Since only two cell cultures for each treatment were harvested at 36 h, except for controls, where four were available, the additional two radioactivities at 27 h were also included in order to obtain more reliable information on changes

Fig. 3. Fluorographs of SDS/polyacrylamide gels

The fluorograph lanes show radioactivity changes in the various bands from individual wells incubated for the indicated times in the presence of 10 mM-3-methyladenine, 100 μg of leupeptin/ml, 20 mM- NH_4Cl , or control cultures. The migration positions of molecular-mass markers are indicated.

with incubation time. For the purpose of grouping data, the changes between 0 h and 27 h were multiplied by 36/27 and the average changes over '36 h' are reported in Table 5.

In control cultures a substantial accumulation of small intermediates occurred, accounting at least in part for the differences between loss of aldolase monomer radioactivity and accumulation of radioactivity in trichloroacetic acid-soluble products. This increase in small intermediates clearly indicates that the initial degradation of NEM-aldolase is not totally rate-limiting, but that a second limiting step must occur between the small intermediates and acid-soluble products. Such a secondary block is not part of most general theories of the regulation of intracellular protein breakdown, although breakdown products have been identified for other proteins both *in vivo* (Reznick *et al.*, 1985; Toda & Ohashi, 1986) and in cultured cells (Chandler & Ballard, 1988). It is also consistent with the observation of 'indigestible contents' detected by electron microscopy within the lysosomal system (Pfeifer, 1987).

Addition of either insulin or 3-methyladenine would be expected to decrease the accumulation of any lysosomal breakdown products, because these inhibitors of autophagy should only decrease the rate of NEM-aldolase uptake into autophagic vacuoles (Pfeifer, 1987; Mortimore, 1987; Seglen, 1987). Results with both insulin and 3-methyladenine were consistent with this inter-

pretation (Table 5), in that a similar proportion (about 40 %) of the radioactivity lost from the aldolase monomer was found in small intermediates when compared with the control. With insulin, inhibition increases with time. Assuming a maximal inhibition of 24 % (Tables 2 and 3), and applying the entire decrease in radioactivity lost from the monomer to the accumulation of small intermediates, we can calculate that radioactivity in this fraction could be as little as $100 \times 4100/11600 = 35\%$ of that lost from the aldolase monomer, a value not significantly less than that calculated above. This correction was therefore not applied to the calculations of Table 5. The greater inhibition of monomer breakdown by 3-methyladenine was also associated with an increased accumulation of large intermediates. Perhaps 3-methyladenine had effects in the HeLa cells other than as an inhibitor of autophagy. At the concentration tested, 10 mM, this drug was somewhat toxic to the HeLa cells, since it was the only agent tested that produced a significant increase in the amount of labelled protein released into the medium.

The results with NH_4Cl support the proposal that part of the 3-methyladenine response might have been an artifact, because this very effective inhibitor of lysosomal proteolysis only decreased NEM-aldolase breakdown by 44 %, compared with 61 % for 3-methyladenine (Table 4). Indeed, the difference between these two treatments was even greater when only the 27 h and 36 h values were used for comparison (Table 5). Ammonia appeared to block the further breakdown of small intermediates when added either alone or in the presence of insulin (Table 5), since the flux through the pool of small intermediates was decreased. For example, the flux into the small-intermediate pool per monolayer was 10200 d.p.m. in the presence of NH_4Cl compared with 15100 d.p.m. in control cultures, whereas the fluxes out were 3700 d.p.m. and 7900 d.p.m. respectively (calculated

from Table 5). Yet approximately the same amount of radioactivity accumulated in the small intermediate pool under both conditions. Ammonia did not increase the amount of radioactivity present in large intermediates.

Leupeptin inhibited the breakdown of NEM-aldolase by approx. 50 % (Tables 4 and 5), somewhat greater than observed with NH_4Cl . This greater effect of leupeptin may be indicative of some minor involvement of neutral cysteine proteinases (calpains; Bond & Butler, 1987) located outside the lysosomal system. These proteinases catalyse only limited bond cleavage (Bond & Butler, 1987) and would be expected to generate peptides of large size. In contrast, the measurements of accumulation of intermediates in the degradation of NEM-aldolase (Table 5) are indicative of leupeptin acting predominantly to inhibit the generation of small aldolase intermediates from large intermediates. Thus we observed increases in the accumulation of large intermediates with leupeptin alone or with leupeptin plus insulin, coupled with dramatic decreases in small intermediates under the same conditions (Table 5).

The results described above are interpreted as ammonia acting predominantly at a later stage in the degradation process than does leupeptin. When these two agents are present together, the overall extent of inhibition is very great (Tables 4 and 5), and both small and large intermediates accumulated (Table 5, line 10). It seems likely that the cysteine cathepsins B, H and L that are inhibited by leupeptin (Kirschke *et al.*, 1980) are involved in early proteolytic processes within the lysosomal system, whereas other proteinases, also with acid pH optima, may be important for the breakdown of smaller intermediates to acid-soluble material. Finally it is clear that loss of a protein band or an enzyme activity cannot always be equated with total degradation of that material to amino acids, since in both the presence and the absence of inhibitors intermediates may accumulate.

Table 5. Effect of inhibitors on the generation of NEM-aldolase breakdown products

Bands detected by fluorography in the same gels as used for the quantification of the aldolase monomer (Table 4) and with smaller molecular masses were cut out and the radioactivity was determined. Values are expressed as total radioactivity lost (–) or gained (+), and are means $\pm$ S.E.M. for determinations at '36 h' as described in Table 3. The numbers of wells are indicated in parentheses.

Treatment	$10^{-2} \times$ Radioactivity (d.p.m.) lost or gained in:		
	Aldolase monomer	Large intermediates (31–38 kDa)	Small intermediates (10–30 kDa)
None (7)	–152 $\pm$ 9	+1 $\pm$ 2	+72 $\pm$ 6
Insulin (1 μM) (4)	–123 $\pm$ 7	+12 $\pm$ 5	+48 $\pm$ 4
3-Methyladenine (10 mM) (4)	–69 $\pm$ 4	+19 $\pm$ 8	+28 $\pm$ 5
Leupeptin (100 $\mu\text{g}/\text{ml}$) (4)	–81 $\pm$ 3	+25 $\pm$ 2	+15 $\pm$ 3
NH_4Cl (20 mM) (3)	–106 $\pm$ 6	+4 $\pm$ 1	+65 $\pm$ 2
Insulin, 3-methyladenine (3)	–94 $\pm$ 4	+23 $\pm$ 8	+44 $\pm$ 6
Insulin, leupeptin (3)	–69 $\pm$ 1	+30 $\pm$ 4	+4 $\pm$ 2
Insulin, NH_4Cl (4)	–115 $\pm$ 6	+20 $\pm$ 7	+71 $\pm$ 7
3-Methyladenine, leupeptin (4)	–59 $\pm$ 2	+13 $\pm$ 4	+23 $\pm$ 3
Leupeptin, NH_4Cl (4)	–57 $\pm$ 7	+12 $\pm$ 6	+28 $\pm$ 7
Insulin, leupeptin, NH_4Cl (4)	–64 $\pm$ 6	+10 $\pm$ 8	+27 $\pm$ 8

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