Selective Control of the Degradation of Normal and Aberrant Proteins in Reuber H35 Hepatoma Cells

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1. Rates of degradation of normal and abnormal protein were measured in hepatoma cells after labelling first for 16 h with [^{14}C]leucine plus L-arginine and then for 3 h with [^3H]leucine plus the arginine analogue, L-canavanine. 2. Over the first 2 h of the degradation period, canavanine-containing proteins were degraded at approximately 5 times the average degradation rate of normal proteins. 3. Degradation of normal proteins was inhibited by about 30% by insulin, cycloheximide, puromycin, leupeptin, antipain and foetal calf serum, whereas these agents had a negligible effect on the breakdown of canavanine-containing proteins. 4. Other compounds inhibited degradation of both classes of protein to equal extents. 5. Combination experiments showed no additional inhibitory effects on the degradation of normal proteins over degradation measured in the presence of a single selective inhibitor. 6. In contrast with the results with a 16 h labelling period, the degradation of normal proteins labelled for only 3 h was not inhibited by insulin. 7. These results are explained by a model with two distinct pathways of protein turnover. The first of these pathways involves the formation of autophagic vacuoles and would be completely inhibited by each of the selective inhibitors. Normal and canavanine-containing proteins would be catabolized by this pathway at equal rates. We propose that degradation by a second pathway is not regulated by the agents tested, but by the inherent stability of each protein.

Isotope-incorporation experiments with *Escherichia coli* and with mammalian cells have shown that structural analogues of the normal amino acid constituents of proteins may be incorporated into new protein chains. Such proteins are rapidly degraded within the cell (Pine, 1967; Goldberg, 1972; Knowles *et al.*, 1975*a,b*), a process that both restricts the accumulation of abnormal protein and provides a mechanism for the re-utilization of the constituent amino acids. The abnormal amino acids may also be re-utilized in a second cycle of protein synthesis, but since the respective tRNA-acylating enzymes usually have a lower affinity for the incorrect than for the correct amino acids, a dilution of the abnormal amino acid by the 'correct' structural analogue will restrict the incorporation of abnormal amino acids to conditions in which they are present in high concentrations (see Goldberg & Dice, 1974).

To remove incorrect proteins efficiently from a cell it is important that the proteolytic degradation process is independent of physiological controls that regulate normal protein turnover. In the present report we have shown that many agents that inhibit overall protein degradation do not decrease the catabolism of abnormal proteins.

Materials and Methods

Chemicals

Cycloheximide, puromycin, L-canavanine, 7-amino-1-chloro-3-tosylamidoheptan-2-one (TLCK), 1-chloro-4-phenyl-3-tosylamidobutan-2-one (TPCK), phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor, lima-bean trypsin inhibitor, ox pancreas trypsin inhibitor, theophylline, dibutyl cyclic AMP, and the amino acids and vitamins required to prepare culture media were from Sigma Chemical Co., St. Louis, MO, U.S.A. L-[4,5- ^3H]leucine (specific radioactivity 50–60 Ci/mmol), L-[U- ^{14}C]leucine (specific radioactivity 300–350 mCi/mmol) and $\text{NaH}^{14}\text{CO}_3$ were from The Radiochemical Centre, Amersham, Bucks., U.K. The sources of tissue-culture dishes, antibiotics and reagents have been described previously (Knowles *et al.*, 1975*b*). Leupeptin, pepstatin and antipain were kindly provided by Dr. H. Umezawa, Microbial Chemistry Research Foundation, 14-23, Kamiosaki 3-Chome, Shinagawa-Ku, Tokyo, Japan. The insulin used (Actrapid) was a product of Novo Industri A/S, Copenhagen, Denmark.

Measurement of protein degradation

Reuber H35 hepatoma cells (Reuber, 1961) adapted to tissue culture (Pitot *et al.*, 1964) were grown as monolayers in Petri dishes under sterile conditions at 37°C (Knowles *et al.*, 1975b). The procedures used for the measurement of protein degradation in the cells are identical with those given under 'Type 1 experiment' by Knowles *et al.* (1975b), except for the omission of dibutyryl cyclic AMP, theophylline and dexamethasone in all experiments except those listed in Table 5, and for other modifications specifically mentioned in the text. Briefly, the technique was to label cell proteins for 16h in the presence of [¹⁴C]-leucine and arginine; replace the medium with one containing [³H]leucine and 0.5mM-canavanine, but no arginine; incubate for a further 3h; rinse the monolayers three times with a salts solution containing 2mM-leucine; add standard degradation medium, which consisted of Earle's salts, the vitamins present in Eagle's Minimal Essential Medium (Eagle, 1959) and 2mM-leucine together with the other compounds listed for each experiment; and leave for a degradation period that was usually 2h before harvesting. Radioactivity in amino acids in the medium and the cells and in cell proteins were determined as trichloroacetic acid-soluble or -insoluble material as described by Knowles *et al.* (1975b).

Degradation is expressed as the percentage degradation, which is 100 times the radioactivity in ³H-labelled or ¹⁴C-labelled amino acids divided by the total ³H or ¹⁴C radioactivity respectively. The effects of potential inhibitors on protein degradation are presented as the percentage inhibition of the average degradation rate of either ¹⁴C-labelled arginine-containing protein or ³H-labelled canavanine-containing protein, compared in each case with catabolism in control medium. The percentage inhibition is calculated as:

100 ×

$$\left(\frac{\% \text{ degradation, control} - \% \text{ degradation, test}}{\% \text{ degradation, control} - \% \text{ degradation at 0h}} \right)$$

Degradation of phosphoenolpyruvate carboxykinase (GTP)

Degradation of ³H-labelled canavanine-containing enzyme and ¹⁴C-labelled arginine-containing enzyme was measured in a manner identical with that described for a 'Type 1 experiment' by Knowles *et al.* (1975a,b). The degradation medium was Eagle's Minimal Essential Medium (Eagle, 1959) supplemented with 2mM-leucine and the additions listed in Table 5. The first-order degradation rate constant for phosphoenolpyruvate carboxykinase (GTP) was calculated from the formula:

$$-\frac{dE}{dt} = k_d(E_t) - k_s$$

where $-dE/dt$ is the rate of decrease in enzyme radioactivity, k_d is the degradation rate constant, E_t is the enzyme radioactivity at the time t and k_s is the zero-order rate constant for the synthesis of radioactive enzyme. This last component was taken as zero, since it is assumed that re-incorporation is effectively prevented by the leucine chase.

We have calculated the degradation rate constant and the standard error of this number for the loss of enzyme radioactivity (expressed as d.p.m. per mg of protein) by a least-squares program prepared by M. C. Childs for use with the CSIRO Control Data 7600 Computer. The values used were from ten separate Petri dishes with cells harvested at 0, 1, 2, 3 and 4h.

Protein measurements

Protein in lysed cell fractions (Knowles *et al.*, 1975b) was measured as described by Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Results

The degradation of normal proteins in Reuber cells occurs at a rate of approx. 3%/h and is somewhat inhibited by the inclusion of foetal calf serum in the degradation medium (Fig. 1). This effect indicates that protein catabolism is low during growth conditions, whereas the cessation of growth is accompanied by an increased rate of degradation. Proteins synthesized in the same cells in the presence of canavanine and [³H]leucine are degraded at a much faster rate than normal proteins over the initial 2h of the degradation period (Fig. 1). In contrast with the effects on normal protein degradation, the addition of foetal calf serum did not decrease the degradation of canavanine-containing protein.

For further investigations into the regulation of the degradation of ¹⁴C-labelled arginine-containing proteins and ³H-labelled canavanine-containing proteins we have replaced foetal calf serum by a defined medium containing amino acids and insulin. These conditions similarly result in an inhibition of normal protein degradation and have a minimal effect on the more rapid breakdown of canavanine-containing proteins (Table 1). It is apparent that although the addition of amino acids results in only a slight decrease in the degradation of arginine-containing proteins, insulin gives a large effect whether or not amino acids are included in the medium.

The experiment in Table 1 represents a comparison between the degradation of normal proteins labelled over a 16h period with canavanine-containing proteins labelled for only 3h. In addition, the experimental design of the double-label study requires the

Fig. 1. Effect of foetal calf serum on protein degradation

Degradation of (a) ^{14}C -labelled arginine-containing proteins and (b) ^3H -labelled canavanine-containing proteins in Reuber hepatoma cells. Protein degradation was measured as described in the Materials and Methods section and is expressed as the percentage degradation of each class of protein. Control cells (●) had Eagle's Minimal Essential Medium (Eagle, 1959) plus 2mM-L-leucine present during the degradation period, and 10% (v/v) foetal calf serum was a further addition to other cells (○). Values are means $\pm$ S.E.M. for three dishes of cells at each time-point.

omission of [^{14}C]leucine during the 3h period in which canavanine-containing proteins are being synthesized in the presence of [^3H]leucine. During this 3h period any labile normal proteins will be degraded, so that a measurement of the degradation of ^{14}C -labelled protein is biased towards more stable proteins. A lack of insulin inhibition may also occur if the degradation of labile but otherwise normal proteins is followed. Table 2 (line 3) shows that this interpretation is indeed true, since the degradation of normal proteins labelled for only 3h and measured without an intervening chase is only minimally decreased by insulin. The absolute degradation rates in this experiment are greater than found for normal proteins in the standard double-label experiment (line 2) or for normal proteins labelled for 16h with a subsequent 3h chase in the absence of canavanine (line 1). However, the degradation of briefly labelled normal proteins is still less than half as rapid as for canavanine-containing proteins (line 4).

In all other experiments reported here we have compared the effects of various compounds on the degradation of 16h-labelled arginine-containing proteins and 3h-labelled canavanine-containing proteins. This experimental procedure permits measurements of degradation of normal protein and canavanine-containing protein in the same cells at the same time, with a corresponding decrease in variability.

The effects of a range of insulin concentrations on the degradation of arginine-containing proteins and canavanine-containing proteins were measured over a 2h period (Fig. 2). Insulin does not inhibit the degradation of canavanine-containing proteins at any concentration tested. However, insulin concentrations between 10pM and 0.5 μM decrease the degradation of normal proteins by 25–30%. The effect is lost at an insulin concentration of 1pM. It should be noted that the effective concentrations of insulin are within the physiological range for rat blood (Girard *et al.*, 1973).

Table 1. Effects of amino acids and insulin on protein degradation

Percentage degradation of ^{14}C -labelled arginine-containing proteins and ^3H -labelled canavanine-containing proteins was measured in hepatoma cells. Details of the procedures involved are given in the Materials and Methods section. When added to the standard degradation medium, insulin was present at 7 nM and amino acids were at twice the concentrations in Eagle's Minimal Essential Medium (Eagle, 1959). Values are means $\pm$ S.E.M. for six determinations.

Degradation period (h)	Additions	Percentage degradation of	
		Canavanine-containing proteins	Arginine-containing proteins
0	—	5.9 $\pm$ 0.2	1.35 $\pm$ 0.21
2	—	27.3 $\pm$ 0.6	6.10 $\pm$ 0.12
2	Insulin	25.7 $\pm$ 0.4	4.52 $\pm$ 0.04
2	Amino acids	27.4 $\pm$ 0.4	5.60 $\pm$ 0.13
2	Insulin+amino acids	26.6 $\pm$ 0.6	4.59 $\pm$ 0.09

Table 2. *Effects of different labelling periods on the subsequent degradation rate*

Protein synthesized in cells was labelled as described in the Materials and Methods section except for the modifications listed under 'Labelling conditions' and 'Chase conditions' below. In each experiment [^3H]leucine was added with either 0.5 mM-arginine or 0.5 mM-canavanine to the cells in arginine-free Eagle's Minimal Essential Medium (Eagle, 1959) for the times given below. At the end of the labelling period, degradation medium was either added directly (no chase) or after an intermediate 3 h incubation in medium similar to that used for labelling, but with [^3H]leucine omitted and 0.5 mM-arginine or 0.5 mM-canavanine present. Degradation was measured over 2 h and was corrected for the apparent degradation at zero time (see '0h' in Table 1). Where added, insulin was present at 7 nM. Values are means $\pm$ S.E.M. for eight determinations.

Labelling conditions	Chase conditions	Percentage degradation in 2 h with		Inhibition by insulin (%)
		Salts	Salts+insulin	
Arginine, 16h	Arginine, 3h	5.33 $\pm$ 0.27	4.07 $\pm$ 0.12	24 $\pm$ 1
Arginine, 16h	Canavanine, 3h	5.74 $\pm$ 0.12	3.38 $\pm$ 0.06	28 $\pm$ 3
Arginine, 3h	No chase	9.93 $\pm$ 0.40	9.47 $\pm$ 0.27	5 $\pm$ 2
Canavanine, 3h	No chase	21.4 $\pm$ 0.3	19.9 $\pm$ 0.4	7 $\pm$ 1

Fig. 2. *Effect of insulin on protein degradation*

The percentage inhibition of the degradation of canavanine-containing proteins (○) and arginine-containing proteins (●) over a 2 h period at different insulin concentrations. Standard degradation medium was used. Values are the means $\pm$ S.E.M. for between four and 12 determinations at each insulin concentration.

Several proteolytic inhibitors restrict the degradation of proteins within intact Reuber cells (Tables 3 and 4). The bacterial cathepsin B inhibitors, leupeptin and antipain, selectively inhibit the degradation of normal proteins, a property also shared by 7-amino-1-chloro-3-tosylamidoheptan-2-one. Although these three compounds are also trypsin inhibitors (Huisman *et al.*, 1974; Suda *et al.*, 1972; Aoyagi *et al.*, 1969; Shaw, 1967), other inhibitors such as phenylmethanesulphonyl fluoride and the trypsin inhibitors from ox pancreas, lima bean and soya bean have no effect on the degradation of normal or abnormal proteins. This distinction could have been produced by the failure of the latter group of inhibitors either to penetrate the cell to the site of proteolysis or to act on cathepsin B.

The chymotrypsin inhibitor, 1-chloro-4-phenyl-3-tosylamidobutan-2-one, inhibits the degradation of arginine-containing proteins and canavanine-containing proteins to an equivalent extent (Table 3), and at concentrations above 0.2 mM it completely inhibits protein degradation and causes the cells to become detached from the Petri dish. Pepstatin, which is an inhibitor of cathepsin D at concentrations below 10 ng/ml (Aoyagi *et al.*, 1972), had no inhibitory action on protein degradation in Reuber cells at a 1000-fold higher concentration (Table 4).

Several other compounds were tested for their action on protein catabolism in Reuber cells (Tables 3 and 4). The metabolic poisons NaF and dinitrophenol inhibit the breakdown of normal proteins and canavanine-containing proteins to an equal extent, whereas NaN₃ shows a partially selective effect on normal proteins. The two inhibitors of protein synthesis tested, cycloheximide and puromycin, are selective inhibitors of normal protein degradation over wide concentration ranges. Selective effects on the degradation of normal proteins were found with NH₄Cl and, as mentioned above, with foetal calf serum and insulin.

Insulin, cycloheximide, NH₄Cl and leupeptin would be expected to affect cell metabolism in different and unrelated ways, although each compound inhibits the breakdown of normal but not abnormal proteins. Accordingly, we have used combinations of these reagents to see if any additive effects on protein degradation can be demonstrated (Table 5). We find that although insulin, leupeptin and cycloheximide individually inhibit the breakdown of arginine-containing proteins by about 30%, there are no additive effects. NH₄Cl, at the concentration chosen in these experiments, is a more effective inhibitor of normal protein breakdown, although the catabolism of canavanine-containing proteins is also decreased. However, no further effect on the degradation of

Table 3. *Inhibition of protein degradation in serum-free medium*

Values represent means $\pm$ S.E.M. for the number of experiments indicated in parentheses and are expressed as the percentage inhibition of the degradation of arginine-containing proteins and canavanine-containing proteins in the test incubations as compared with controls where cells were incubated for 2 h in Eagle's Minimal Essential Medium (Eagle, 1959) with 2 mM-leucine added. A negative value indicates stimulation of degradation.

Additions	Concentration	Inhibition (%) of the degradation of	
		Canavanine-containing proteins	Arginine-containing proteins
Phenylmethanesulphonyl fluoride (4)	1 mM	-5 $\pm$ 4	-5 $\pm$ 11
7-Amino-1-chloro-3-tosylamidoheptan-2-one (7)	0.1 mM	13 $\pm$ 2	30 $\pm$ 4
7-Amino-1-chloro-3-tosylamidoheptan-2-one (4)	0.5 mM	21 $\pm$ 2	51 $\pm$ 9
1-Chloro-4-phenyl-3-tosylamidobutan-2-one (6)	0.05 mM	16 $\pm$ 3	17 $\pm$ 5
1-Chloro-4-phenyl-3-tosylamidobutan-2-one (4)	0.1 mM	45 $\pm$ 8	55 $\pm$ 3
Lima-bean trypsin inhibitor (4)	0.5 mg/ml	-4 $\pm$ 8	-7 $\pm$ 5
Soya-bean trypsin inhibitor (4)	0.5 mg/ml	5 $\pm$ 4	7 $\pm$ 2
Ox pancreas trypsin inhibitor (4)	0.1 mg/ml	-12 $\pm$ 4	-4 $\pm$ 3
NaF (6)	2 mM	18 $\pm$ 1	19 $\pm$ 3
NaN ₃ (7)	2 mM	12 $\pm$ 3	19 $\pm$ 2
Dinitrophenol (4)	0.1 mM	33 $\pm$ 6	39 $\pm$ 7
EDTA (4)	10 mM	3 $\pm$ 8	6 $\pm$ 4
Cycloheximide (7)	0.01 mM	8 $\pm$ 3	22 $\pm$ 4
Cycloheximide (8)	0.1 mM	9 $\pm$ 3	30 $\pm$ 4
Cycloheximide (8)	1 mM	11 $\pm$ 3	32 $\pm$ 3
Puromycin (8)	5 μ M	0 $\pm$ 2	11 $\pm$ 4
Puromycin (8)	0.05 mM	7 $\pm$ 2	29 $\pm$ 4
Puromycin (8)	0.5 mM	13 $\pm$ 2	35 $\pm$ 4
Insulin (20)	7 nM	3 $\pm$ 2	26 $\pm$ 1

Table 4. *Inhibition of protein degradation by various compounds*

Values represent means $\pm$ S.E.M. for the number of experiments indicated in parentheses and are expressed as the percentage inhibition of the degradation of arginine-containing proteins and canavanine-containing proteins in the test incubations as compared with control cells incubated in the presence of standard degradation medium for 2 h. Amino acids were added at twice (mixture A) or five times (mixture B) the concentration in Eagle's Minimal Essential Medium (Eagle, 1959).

Additions	Concentration	Inhibition (%) of the degradation of	
		Canavanine-containing proteins	Arginine-containing proteins
NaN ₃ (9)	10 mM	34 $\pm$ 3	50 $\pm$ 4
Amino acids, mixture A (16)	—	0 $\pm$ 2	7 $\pm$ 2
Amino acids, mixture B (6)	—	1 $\pm$ 2	8 $\pm$ 2
Leupeptin (6)	50 μ g/ml	10 $\pm$ 3	40 $\pm$ 3
Antipain (6)	50 μ g/ml	3 $\pm$ 1	28 $\pm$ 2
Pepstatin (6)	10 μ g/ml	2 $\pm$ 1	8 $\pm$ 4
NH ₄ Cl (3)	1 mM	5 $\pm$ 2	20 $\pm$ 4
NH ₄ Cl (3)	2 mM	8 $\pm$ 2	32 $\pm$ 3
NH ₄ Cl (3)	5 mM	11 $\pm$ 1	43 $\pm$ 4
NH ₄ Cl (3)	10 mM	18 $\pm$ 2	47 $\pm$ 2

normal proteins is found when either insulin or cycloheximide is added to the cells incubated in the presence of NH₄Cl.

Since insulin, foetal calf serum and other agents inhibit the degradation of normal proteins, but not the unstable canavanine-containing proteins, we decided to examine their effects on the turnover of a rapidly

degraded normal protein, such as phosphoenolpyruvate carboxykinase (GTP). We find that insulin, foetal calf serum and 7-amino-1-chloro-3-tosylamidoheptan-2-one have marginal effects on the degradation rate constant for the normal enzyme (Table 6). The degradation of canavanine-containing enzyme occurs at a much greater rate than that of

Table 5. *Combined effects of inhibitors on protein degradation*

Values have been determined as described in the text and the legend of Table 3 and represent means $\pm$ s.e.m. for the number of experiments in parentheses. Inhibitors, when present, were added at the following concentrations: insulin, 7 nM; cycloheximide, 0.1 mM; NH_4Cl , 5 mM; leupeptin, 50 $\mu\text{g}/\text{ml}$.

Additions	Inhibition (%) of the degradation of	
	Canavanine-containing proteins	Arginine-containing proteins
Insulin (9)	5 $\pm$ 1	28 $\pm$ 2
Cycloheximide (8)	9 $\pm$ 2	27 $\pm$ 3
Insulin + cycloheximide (5)	12 $\pm$ 3	30 $\pm$ 2
NH_4Cl (11)	11 $\pm$ 2	44 $\pm$ 2
NH_4Cl + insulin (7)	14 $\pm$ 3	43 $\pm$ 3
NH_4Cl + cycloheximide (8)	18 $\pm$ 1	43 $\pm$ 4
Leupeptin (11)	9 $\pm$ 2	33 $\pm$ 3
Leupeptin + insulin (8)	7 $\pm$ 2	34 $\pm$ 1
Leupeptin + cycloheximide (7)	11 $\pm$ 2	34 $\pm$ 2

Table 6. *Degradation of phosphoenolpyruvate carboxykinase (GTP)*

Phosphoenolpyruvate carboxykinase (GTP) was induced in hepatoma cells in the presence of [^{14}C]leucine and arginine and subsequently in the presence of [^3H]leucine and 0.5 mM-canavanine before the measurement of enzyme degradation. Control cells were incubated in Eagle's Minimal Essential Medium (Eagle, 1959) plus 2 mM-leucine during the degradation period of up to 4 h. Five separate experiments are reported, each with control cells. Values of the first-order degradation rate constants were calculated as described in the Materials and Methods section.

Additions	Degradation rate constants (h^{-1})	
	Canavanine-containing enzyme	Arginine-containing enzyme
None	0.178 $\pm$ 0.014	0.055 $\pm$ 0.011
Insulin, 7 nM	0.206 $\pm$ 0.012	0.025 $\pm$ 0.013
None	0.143 $\pm$ 0.026	0.056 $\pm$ 0.029
Insulin, 7 nM	0.148 $\pm$ 0.025	0.046 $\pm$ 0.027
None	0.260 $\pm$ 0.017	0.076 $\pm$ 0.024
Foetal calf serum, 10% (v/v)	0.266 $\pm$ 0.015	0.113 $\pm$ 0.020
None	0.144 $\pm$ 0.020	0.057 $\pm$ 0.031
Foetal calf serum, 10% (v/v)	0.161 $\pm$ 0.018	0.075 $\pm$ 0.021
None	0.236 $\pm$ 0.027	0.089 $\pm$ 0.030
7-Amino-1-chloro-3-tosylamidoheptan-2-one, 0.25 mM	0.140 $\pm$ 0.036	0.024 $\pm$ 0.040

normal phosphoenolpyruvate carboxykinase (GTP), and again there is no consistent action of insulin, foetal calf serum or 7-amino-1-chloro-3-tosylamidoheptan-2-one.

Discussion

The degradation of normal proteins is 30% inhibited by optimal concentrations of insulin, puromycin, cycloheximide, foetal calf serum, NH_4Cl , leupeptin, antipain and 7-amino-1-chloro-3-tosylamidoheptan-2-one. Under these conditions the breakdown of canavanine-containing proteins is either not affected or only slightly inhibited. Since the selective inhibitors include hormonal agents, protein-synthesis inhibitors and proteolysis inhi-

bitors, and do not have an apparent common site of action, it was surprising that additive effects were not found when combinations of the selective inhibitors were tested. For example, insulin, the protein-synthesis inhibitor cycloheximide and the cathepsin B inhibitor leupeptin had equivalent effects individually or when tested together.

We propose that the most likely explanation for these findings is an action of all the selective inhibitors to inhibit completely or inactivate a proteolytic pathway that accounts for 30% of the total protein degradation under the conditions tested. This pathway is depicted as pathway A in Scheme 1. The inactive pathway in cells incubated in the presence of insulin or serum becomes activated on the nutritional step-down caused by the removal of these substances from the

Scheme 1. Scheme for protein degradation

The various pathways and regulatory controls are discussed in the text.

medium. Further, we suggest that proteins of short half-life and proteins of long half-life are degraded at approximately equal rates by the pathway. Such a restriction will account for the minimal effects of insulin, cycloheximide and the other selective inhibitors on the breakdown of canavanine-containing proteins. These proteins are very labile and are degraded in the Reuber cells at approximately 5 times the degradation rate of normal proteins (Table 1), so that the percentage contribution of pathway A to the catabolism of canavanine-containing proteins would be approximately one-fifth of 30%, i.e. 6%. This value is close to the observed percentage inhibition of degradation of canavanine-containing proteins that is produced by the selective inhibitors (see Tables 3, 4 and 5). Although an alternative suggestion is for the abnormal proteins to be refractory towards catabolism by pathway A, it is difficult to imagine how the appropriate selection would occur in the cell. A similar difficulty is encountered with labile normal proteins, since these would also need to be resistant to pathway A.

Our hypothesis that about 30% of total protein degradation occurs via a pathway that can be inhibited by a variety of agents and which shows little specificity towards individual proteins can be equated with cellular autophagy. Autophagic vacuoles form by an engulfing process in which cell constituents are surrounded first by a double membrane and eventually form a digestive vacuole by fusing with a primary lysosome (Ashford & Porter, 1962; Hruban *et al.*, 1963; Ericsson, 1969; Arstila *et al.*, 1972). The pro-

teins within the vacuole are then degraded by lysosomal cathepsins. The proposal that short-half-life and long-half-life proteins are degraded at approximately equal rates can be explained by the known properties of the autophagic system. Thus autophagic vacuoles contain mitochondria and other cell constituents (Ashford & Porter, 1962; Ericsson, 1969; Arstila *et al.*, 1972) and would not be expected to select for either short- or long-half-life proteins. Further, insulin is known to prevent the formation of autophagic vacuoles (Jefferson *et al.*, 1974), and indirect experiments suggest that cycloheximide has a similar effect (Neely *et al.*, 1974). Selective inhibition by leupeptin and antipain can also be explained if the lysosomal membrane is impermeable to these cathepsin B inhibitors, since the inhibitors would be engulfed into new autophagic vacuoles along with normal cell material.

If about 30% of protein turnover can be accounted for by degradation in autophagic vacuoles, how is the remaining protein degraded? Recent work in this and other laboratories has shown that enzymes are inactivated in liver extracts at rates that are approximately proportional to their degradation rates *in vivo* (Bond, 1971; Hopgood & Ballard, 1974; Salinas *et al.*, 1974). This inactivation appears to be carried out by microsomal membranes and is accelerated by disulphide compounds such as cystine or oxidized glutathione (Ballard *et al.*, 1974; Ballard & Hopgood, 1976). Proteolysis is not involved (Ballard & Hopgood, 1976). We suggest that protein breakdown initiated by denaturation (depicted as pathway B1 in

Scheme 1) would reflect the inherent stability of a protein molecule as defined by the amino acid sequence and modulated by ligands. By this system short-half-life proteins such as canavanine-containing proteins would be rapidly denatured, whereas proteins with long half-lives would be stable. If the rate of denaturation is greater than the subsequent proteolytic steps (pathway B2, Scheme 1) the model proposes an accumulation of denatured protein in the cell. This would only occur with rapidly denatured proteins and has indeed been demonstrated in canavanine-treated *E. coli* by both centrifugal (Prouty & Goldberg, 1972) and electron-microscopic experiments (Prouty *et al.*, 1975). It has also been suggested that some erythrocyte inclusions reported in human diseases may represent denatured protein (Goldberg & Dice, 1974). In order to explain the selective effects of leupeptin and antipain on protein degradation, it is necessary to propose that either the proteolytic steps in pathway B are catalysed by proteinases other than cathepsin B or there is a separation of the proteinases from the inhibitors. Although no definite explanation can be offered at the present time, it is known that proteinases do occur outside the lysosomes in mammalian cells, with a considerable activity in the microsomal fraction (Bohley *et al.*, 1971), whereas experiments *in vitro* show that inactivated enzymes become attached to the microsomal membranes (Ballard & Hopgood, 1976). Perhaps the proteolytic steps in pathway B (Scheme 1) do not involve lysosomes.

An alternative and simpler explanation of our results can be offered. We used an experimental design that required a period of 3 h between the removal of [^{14}C]leucine and the beginning of the degradation measurements, a time which was necessary for the labelling of canavanine-containing proteins. If the proteolytic degradation within vacuoles has a half-life of several hours, then some of the ^{14}C -labelled protein within the vacuoles will be in a later stage of degradation than the ^3H -labelled protein. If insulin and the selective inhibitors have no effect on pre-formed autophagic vacuoles, but prevent the formation of new vacuoles, a greater apparent effect on the degradation of ^{14}C -labelled normal proteins will be seen. It might therefore be possible to explain our results by a single catabolic pathway carried out in autophagic vacuoles. We do not believe this hypothesis can account for our findings since: (1) it would require a progressively greater inhibition of protein degradation with time, a condition not found with the selective inhibitors (Fig. 1; see also Poole & Wibo, 1973); (2) autophagic vacuoles would need to be present under all nutritional states, with only a slight increase during step-down conditions; (3) in order to explain the very rapid degradation of canavanine-containing proteins it would be necessary for half of the cell proteins to be engulfed every 2–3 h by

organelles that occupy only 0.5% of the cell volume (if liver can be taken as a guide; Beaufay, 1969).

Two examples of proteins with rapid turnover rates have been investigated in the present study, canavanine-containing proteins and phosphoenolpyruvate carboxykinase (GTP). We were not able to show any consistent effect of the selective inhibitors on the degradation of phosphoenolpyruvate carboxykinase (GTP), possibly owing to technical limitations of the methods used. We followed the loss of radioactivity in the enzyme pool, so that only large changes in catabolic rate would be readily detected. Degradation of canavanine-containing proteins was followed by the increase in amino acid radioactivity with time, starting from a very low amount of radioactive amino acid. This method is far more sensitive to changes in degradation rate than that required for phosphoenolpyruvate carboxykinase (GTP).

Experiments with fibroblasts have shown that the degradation of protein with short half-lives is not susceptible to inhibition by the addition of fresh medium to the culture (Poole & Wibo, 1973), whereas the breakdown of proteins with long half-lives is decreased. Such a finding agrees with the data reported in Table 2 and is consistent with the concept discussed here of two distinct pathways of protein degradation.

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