A comparison of lysosomal involvements in the degradation of normal and abnormal endogenous proteins of differing half-lives in MRC5 cells

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Protein degradation by diploid human-embryo lung fibroblasts (MRC5 cells) in monolayer culture was studied. 1. Varying the labelling period of proteins was found to alter the half-lives of labelled abnormal canavanine-containing proteins to an extent very similar to that obtained with normal proteins. 2. By manipulating the times of labelling it was possible to generate a species of abnormal protein with a greater half-life than that of a species of normal protein. A comparison of the lysosomal involvement in their degradation as determined both by inhibition by methylamine, a lysosomotropic agent, and by the degree of increase in protein degradation in step-down conditions, indicated that the degree of lysosomal involvement was not entirely dependent upon the half-life of the protein, but that abnormal proteins are preferentially degraded non-lysosomally. 3. The microtubule inhibitors colchicine and vinblastine were found to stimulate statistically basal protein degradation of normal long-labelled protein, whereas they had less effect upon the basal degradation of the other species of proteins studied and very little effect upon step-down degradation of all proteins studied. The stimulation in protein degradation found did not seem to involve the acid proteinases of lysosomes.

Over the past decade, many studies have been made of the mechanisms of eukaryotic protein degradation [reviewed by Goldberg & Dice (1974), Goldberg & St. John (1976), Dean (1980) and Amenta & Brocher (1981)]. The use of specific proteinase inhibitors and reagents that inhibit lysosomal function suggest that, in cells incubated with sufficient nutrients to permit cell growth and where protein degradation is at a basal level, there are at least two pathways of protein degradation: a lysosomal pathway involving proteinases known to be present in these organelles (Barrett, 1980), and a cytosolic pathway, accounting for 70–80% of protein degradation, which involves neutral proteinases. Some of the latter have been identified in various cell types (Reville *et al.*, 1976; De Martino & Goldberg, 1979).

When isolated cells or organ cultures are placed in step-down conditions, i.e. in amino acid- or serum-deficient medium (nutrient-deficient medium), the amount of degradation by the lysosomal pathway is increased. It is not clear whether this is by the same mechanism as that occurring in basal degradation (Amenta & Brocher, 1981; Poole *et al.*, 1978),

because, for example, the lysosomal contribution to basal degradation is not inhibited by insulin, whereas the induced proteolysis in step-down conditions is (Amenta & Brocher, 1981). Recent studies (Kovács *et al.*, 1981; Schworer *et al.*, 1981) using stereological analyses of autophagic vacuoles have suggested that the increase in lysosomal protein degradation in step-down conditions is due to an increase in autophagy.

Not all proteins are degraded to the same extent by the lysosomal and cytosolic pathway. It has been shown in a variety of cell and tissue cultures that long-lived proteins are more likely to be degraded lysosomally, and their degradation is increased in step-down conditions. By contrast, short-lived proteins, including abnormal proteins with amino acid analogues incorporated into them, are preferentially degraded by the cytosolic pathway. Degradation of short-lived proteins is only slightly increased in step-down conditions (Knowles & Ballard, 1976; Neff *et al.*, 1979), which is evidence that they are preferentially not degraded in lysosomes.

Work on protein degradation has been carried out on a variety of cell types, but one criticism is that, in many of these studies, such as those employing isolated hepatocytes or perfused organs, the cells

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had recently undergone a great change in their environment, so basal protein degradation would be difficult to establish and biological variation between cell samples would be great. To overcome these difficulties we have used a passageable non-transformed cell-culture line for our studies. The cells employed were MRC5 diploid human lung fibroblasts, which do not possess the capability to metabolize the amino acid markers used in degradation studies exhibited by other cells such as liver and muscle cells. Also, our laboratory has previously used these cells in studies on the effects of cell senescence on protein degradation (Dean & Riley, 1978). In the present study we have examined the effects of various agents known to have direct and indirect influences on protein degradation. These include: colchicine and vinblastine, which are inhibitors of microtubule assembly (Dustin, 1978); leupeptin, a thiol-proteinase inhibitor that mainly inhibits lysosomal cathepsin B and cytosolic calcium-activated proteinase (Barrett, 1980); and methylamine, which inhibits lysosomal proteinases by raising the intralysosomal pH above the pH optima of the proteinases (Ohkuma & Poole, 1978). The proteins studied were long-half-life normal proteins and short-half-life normal proteins, which can be distinguished by the length of time of incorporation of radioactive amino acids into them, the former being preferentially labelled over long periods, whereas the latter are preferentially labelled in short pulses (Poole & Wibo, 1973). The effects of the various reagents upon degradation of long-labelled abnormal proteins and short-labelled abnormal proteins, both containing the arginine analogue canavanine, were also studied to see if a similar distinction between routes of degradation exists between these protein types as that documented for normal proteins. To our knowledge this is the first time such a comparison has been reported.

Experimental

Materials

Lactate dehydrogenase assay kit, methylamine, vinblastine sulphate and crystalline bovine insulin were purchased from Sigma Chemical Co., Poole, Dorset, U.K.; colchicine from BDH Ltd., Poole, Dorset, U.K.; leupeptin from Protein Research Foundation, Osaka, Japan; and phosphate-buffered saline tablets (Dulbecco 'A') from Oxoid Ltd., Basingstoke, Hants., U.K. All other chemicals were of analytical purity and were obtained from BDH or Fisons Ltd., Loughborough, Leics., U.K.

All tissue-culture media and sera were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., and Falcon tissue-culture vessels were purchased from Becton-Dickinson Ltd., Wembley, London, U.K. L-[3,4(n)-³H]Valine (sp. radioactivity

57 Ci/mmol), L-[guanido-¹⁴C]arginine monohydrochloride (56 mCi/mmol), [methyl-³H]thymidine (5 Ci/mmol) and [methyl-³H]methylamine hydrochloride (11.7 Ci/mmol) were purchased from Amersham International Ltd., Amersham, Bucks., U.K. DL-[guanido-¹⁴C]canavanine (44.5 mCi/mol) was purchased from Schwartz/Mann, Orangeburg, NY, U.S.A.

Methods

Cell culture. MRC5 diploid human-embryo lung fibroblasts were obtained from Dr. J. P. Jacobs, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London N.W.3, U.K. (Jacobs, 1976). Cells were routinely grown at 37°C in 75 cm² polystyrene culture vessels in 30 ml of minimal essential medium (Eagle's) supplemented with 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, 10% (v/v) foetal-calf serum, and antibiotics (penicillin, 1 k-units/ml, and streptomycin, 1 mg/ml) (hereafter called 'growth medium'). Subcultivation was by trypsin treatment to detach the cells, and half the cells were re-inoculated twice weekly. Cells were routinely checked for fungal, bacterial or mycoplasmic contamination.

Measurement of protein degradation. Cells between passages 30 and 40 were grown to confluence in 24 × 2 cm² Falcon multiwell tissue-culture plates in a humidified CO₂/air (1:19) atmosphere. For experiments using L-[3,4-³H]valine as a cell protein radiolabel, cells were washed three times in valine-free growth medium with 5 min incubations in between. This procedure has been found to deplete cells of free valine (Dean & Riley, 1978). Cells were then incubated with L-[3,4-³H]valine (1 μCi/well) for 18 h (long label) or 30 min (short label) in valine-free medium after which they were washed four times in (a) growth medium containing 10 mM-valine to measure basal degradation or (b) in growth medium lacking serum (step-down medium) containing 10 mM-valine to measure step-down degradation, and then incubated in 1.5 ml (per well) of the same medium. Cells were incubated for 5 min between washes and this procedure has been found to remove virtually all of free radiolabelled valine, and reutilization of L-[3,4-³H]valine was minimal (Dean & Riley, 1978). A 75 μl portion of stock solution of reagents in step-down medium was added to triplicate wells to give the following final concentrations: colchicine, 25 μM; vinblastine sulphate, 10 μM; methylamine, 10 mM; and leupeptin, 50 μg/ml. Portions (200 μl) of culture medium were taken from the triplicate wells at 0, 1, 2.5, 5 and 24 h; these were pooled, and 60 μl of step-down medium or foetal-calf serum were added to make the protein content of cell samples the same. A 75 μl portion of chilled 50% (w/v) trichloroacetic acid was added to

precipitate the protein, and this was removed by centrifugation at 3000g for 30 min. A 500 μ l sample of supernatant was taken and assayed for radioactivity by adding 4 ml of Fiso fluor SC6000 scintillation fluid and counting for radioactivity on an Intertechnique SL30 liquid-scintillation counter. The amount of trichloroacetic acid-precipitable radioactivity in the cell medium was monitored by washing the protein precipitate three times with chilled 5% (w/v) trichloroacetic acid, dissolving in 0.5 ml of 1 M-NaOH and assaying for radioactivity in pH-neutralized samples. Less than 2% of the medium radioactivity was lost during the washing procedure. At the end of the experiment, cells were washed once in phosphate-buffered saline, twice in 5% (w/v) trichloroacetic acid to precipitate macromolecules, and once more in phosphate-buffered saline. The remaining cell protein and DNA was dissolved in 1 M-NaOH and neutralized samples assayed for radioactivity. Less than 5% of cell radioactivity was lost during this washing. The total radioactivity in each set of three wells was therefore calculated from the sum of the radioactivities in each fraction, and the protein degradation was expressed as the percentage of total radioactivity in the system present in the cell medium as a trichloroacetic acid-soluble fraction. Experiments using arginine or canavanine to label the cell proteins were analogous to those above, except that arginine-free medium was used during the labelling and the degradation medium contained 10 mM-arginine. The amounts of radioactive arginine and canavanine added to the cells were 1 μ Ci and 0.6 μ Ci per well respectively.

Monitoring of radiolabelled amino acid breakdown in cell cultures. Cells were treated as in the protein-degradation studies, with the exception that, at the end of the experiment, cells were washed five times in phosphate-buffered saline and not precipitated with trichloroacetic acid. Samples of cell-labelling medium (after an 18 h labelling period), protein-degradation medium (after a 24 h incubation) and cells were freeze-dried, and half of each sample was hydrolysed in 6 M-HCl at 115°C for 18 h. After removing the hydrochloric acid *in vacuo*, hydrolysed and non-hydrolysed samples were run on cellulose t.l.c. plates (0.1 mm thickness; Merck, Darmstadt, Germany) by ascending chromatography in a solvent system of butan-1-ol/acetic acid/water (13:3:3, by vol.). The positions of radioactivity were elucidated by scraping off bands and assaying by liquid-scintillation counting. Identification was by comparison with known reference samples in this solvent system and a system of 100% (w/v)-phenol/NH₃ (200:1, v/v).

Measurement of protein and DNA synthesis. Measurement of DNA and protein synthesis was carried out, correcting for cell number, as described previously (Riley, 1980) utilizing the uptake of

[methyl-³H]thymidine and L-[3,4-³H]valine respectively.

Uptake of methylamine by cells. Cells were grown to confluence in the protein-degradation studies. They were washed three times in growth medium or step-down medium and 1 ml of the same was added per well. A 1.8 μ Ci portion of [methyl-³H]methylamine in sufficient unlabelled methylamine to give a final concentration of 10 mM (the same as was used for protein-degradation studies) was added. Colchicine (25 μ M final concn.), vinblastine sulphate (10 μ M), or step-down medium to serve as control, were added and the cells were incubated a further 24 h at 37°C, after which time they were washed five times in phosphate-buffered saline and dissolved in 0.4 ml of aq. 0.1% (v/v) Triton X-100. The samples were assayed for radioactivity as described above and also for protein by the method of Lowry *et al.* (1951), bovine serum albumin (Cohn fraction V; Koch-Light Ltd., Colnbrook, Bucks., U.K.) being used as the reference. Results are expressed as c.p.m./ μ g of cell protein.

Results

Metabolism of amino acid radiolabels

When L-[3,4-³H]valine was used as the radiolabel in all the samples investigated, radioactivity was only located in the protein (R_F 0) and valine (R_F 0.55) fractions of the chromatogram. On hydrolysing the protein, all the protein radioactivity was transferred to the valine position. In cell-degradation medium, 9% of the radiolabel was in the form of protein resulting from protein secretion from the cells or cell loss from the monolayer. Thus it can be concluded that, apart from incorporation into proteins, negligible metabolism of valine is carried out by MRC5 cells. The results obtained by using L-[guanido-¹⁴C]arginine and DL-[guanido-¹⁴C]canavanine are shown in Table 1. When the samples were hydrolysed, more than 98% of the radioactivity in the protein fractions was transferred to that of arginine or canavanine. It can be seen that ¹⁴C-labelled urea was formed in cultures from arginine and canavanine by an arginase-like activity. However, [¹⁴C]urea was only formed when foetal-calf serum was present, and comparison of the results with those of a cell-free incubation indicates that arginase activity is present in the serum. The serum arginase was not inactivated by heating the serum to 55°C for 30 min.

Basal and step-down protein degradation of normal and abnormal proteins

It was found (S. A. Wharton & P. A. Riley, unpublished work) that the degradation of long- and short-labelled L-[3,4-³H]valine-containing proteins was very similar to those with L-[guanido-¹⁴C]-

Table 1. *Metabolism of arginine and canavanine*

The analyses of unhydrolysed samples were carried out as described under 'Methods'. The results given are the mean of two determinations, with the metabolites being separated in a solvent system of butan-1-ol/acetic acid/water (13:3:5, by vol.). R_F values were: protein, 0; canavanine, 0.13; arginine, 0.27; and urea, 0.42. Abbreviation used: MEM, minimal essential medium.

Incubation conditions	Label ... Product ...	Radioactivity in fractions (%)					
		Arginine			Canavanine		
		Protein	Arginine	Urea	Protein	Canavanine	Urea
Cell labelling							
Arginine-free MEM + 10% foetal-calf serum		11	72	17	9	85	6
Protein degradation							
MEM + 10% foetal-calf serum + 10 mM-arginine		13	86	0.7	14	84	2
MEM + 10 mM-arginine		4.8	95	0.2	—	—	—
Cell fraction							
Cells in growth medium		69	31	0.3	70	28	2
Cells in step-down medium		73	27	0.2	—	—	—
Cell-free incubations							
Arginine-free MEM + 10% foetal-calf serum		0.4	85	15	8	85	7
Arginine-free MEM		0.1	99	0.5	0.5	99	0.2

arginine-containing proteins. Also, addition of 10 mM-arginine to cells with L-[3,4- ^3H]valine-containing proteins or of 10 mM-valine to cells with L-[*guanido*- ^{14}C]arginine-containing proteins had negligible effects upon the degradation of those proteins, as did incubating cells in valine-free medium during labelling with L-[*guanido*- ^{14}C]arginine or in arginine-free medium during labelling with L-[3,4- ^3H]valine. This shows that the results obtained with L-[3,4- ^3H]valine will be the same as those with L-[*guanido*- ^{14}C]arginine, and thus a comparison between L-[3,4- ^3H]valine-containing proteins (normal) and L-[*guanido*- ^{14}C]canavanine-containing proteins (abnormal) is valid and that differences depend upon the effects of the canavanine molecules themselves.

The addition of 100 m-i.u. of insulin/ml was found not to decrease the amount of degradation of long-labelled normal protein in growth medium, whereas it inhibited step-down degradation by 20%. Thus the 10% (v/v) foetal-calf serum is able to suppress protein degradation down to basal levels. Fig. 1 shows the percentage degradation of the four groups of proteins investigated in the present study on growth or step-down medium. In all experiments, release of trichloroacetic acid-precipitable radioactivity from the cells was not more than 10% of the total radioactivity in the medium. Degradation after 5 h and 24 h under basal conditions can be placed in the following order: abnormal short-label proteins > normal short-label proteins > abnormal long-label proteins > normal long-label proteins. When

amounts (not rates) of degradation after 5 and 24 h respectively are considered, the ratios of basal degradation to short-labelled to long-labelled proteins were similar for normal (2.67 and 1.50) and abnormal (2.71 and 1.60) proteins. The ratios of degradation of abnormal to normal proteins for long-labelled (1.62 and 1.40) and short-labelled (1.64 and 1.50) proteins were also similar. This indicates that the influence of labelling periods on degradation is equivalent for normal and abnormal proteins. Normal proteins are more affected than abnormal proteins by placing the cells in step-down conditions. The effect is greatest on long-labelled normal proteins, where, after 5 and 24 h, degradation is increased by 54 and 33% respectively. The corresponding percentage increases in degradation are 13% and 17% for normal short-labelled protein, and only 1.3% and 2.1% for abnormal long-labelled protein. Abnormal short-labelled proteins exhibited a decrease in degradation of 19% and 9.3% after 5 and 24 h respectively in step-down conditions.

Effects of colchicine, leupeptin and methylamine upon DNA and protein synthesis

The reagents were used at the following final concentrations: colchicine, 25 μM ; methylamine, 10 mM; and leupeptin, 50 $\mu\text{g}/\text{ml}$. None of them affected DNA synthesis in growth medium to a greater extent than simply placing cells in step-down medium. Thus, over a 24 h incubation at 37°C, colchicine inhibited synthesis by 35%, methylamine

Fig. 1. Degradation of normal and abnormal proteins in growth and stepdown media

Results are means $\pm$ S.D. for the numbers of determinations shown in parentheses. (a) Normal long-label protein (7); (b) abnormal long-label protein (4); (c) normal short-label protein (4); (d) abnormal short-label protein (4). ●, Growth medium; ○, step-down medium.

by 46% and leupeptin by 23%. Step-down conditions inhibited DNA synthesis by 88%. The effects of the compounds on protein synthesis were proportionately less, being 1, 17 and 10% for colchicine, methylamine and leupeptin respectively. Step-down conditions decreased protein synthesis by 23%. Therefore these reagents had less effect upon macromolecular synthesis than did placing the cells in step-down medium. When viewed by optical microscopy (magnification $\times 200$) it was seen that step-down medium caused cells to become more polarized in shape and less spread out than in growth medium. Leupeptin in basal or step-down medium had no visible effect upon cell morphology com-

pared with controls. Colchicine caused a decrease in polarity of cell shapes in both media, and methylamine caused vacuolation of the cells. These vacuoles were larger and occupied more cell volume in growth medium than step-down medium. None of the reagents caused cells to spontaneously round up or detach from the monolayer. Cell viability, as determined by Trypan Blue exclusion, was not decreased by any of the reagents (S. A. Wharton & P. A. Riley, unpublished work).

Effects of colchicine, leupeptin and methylamine upon protein degradation

Table 2 shows the effect on degradation of the

Table 2. *Percentage of basal and step-down protein degradation in the presence of colchicine, leupeptin and methylamine*
Results are the means for five determinations for long-label normal proteins and four for the other three protein groups. The concentrations of the reagents were: colchicine, 25 μ M; leupeptin, 50 μ g/ml; and methylamine, 10 mM. Results are expressed as means $\pm$ S.D. The probability values (two-tailed) of the significance of differences versus controls calculated by Student's *t* test are designated as follows: $\ddagger P < 0.05$; * $P < 0.001$.

Reagents	Normal proteins				Abnormal proteins			
	Long label		Short label		Long label		Short label	
	5 h	24 h	5 h	24 h	5 h	24 h	5 h	24 h
(a) Growth medium								
Control	8.6 $\pm$ 0.4	29.6 $\pm$ 1.0	22.9 $\pm$ 2.3	44.5 $\pm$ 3.1	13.9 $\pm$ 0.2	41.5 $\pm$ 3.0	37.6 $\pm$ 3.3	66.6 $\pm$ 3.7
Colchicine	9.5 $\pm$ 0.4*	34.5 $\pm$ 2.0*	23.1 $\pm$ 2.2	46.7 $\pm$ 3.9	14.9 $\pm$ 0.4	43.2 $\pm$ 2.2	36.8 $\pm$ 4.7	68.2 $\pm$ 3.8
Leupeptin	8.1 $\pm$ 0.5	26.8 $\pm$ 1.9 $\ddagger$	21.5 $\pm$ 1.5	40.9 $\pm$ 1.3	13.6 $\pm$ 0.7	36.9 $\pm$ 1.4	33.5 $\pm$ 5.6	59.3 $\pm$ 4.4
Methylamine	6.7 $\pm$ 0.8*	22.3 $\pm$ 3.3*	18.8 $\pm$ 1.7	35.5 $\pm$ 2.3 $\ddagger$	12.1 $\pm$ 0.7	35.2 $\pm$ 1.0	32.9 $\pm$ 2.4 $\ddagger$	58.5 $\pm$ 2.1
(b) Step-down medium								
Control	13.0 $\pm$ 1.6	39.2 $\pm$ 1.4	26.0 $\pm$ 1.1	52.0 $\pm$ 2.8	14.1 $\pm$ 0.9	42.4 $\pm$ 1.0	30.6 $\pm$ 4.1	67.0 $\pm$ 3.3
Colchicine	12.7 $\pm$ 0.9	40.4 $\pm$ 1.9	28.2 $\pm$ 1.1	50.5 $\pm$ 2.3	14.8 $\pm$ 0.5	42.9 $\pm$ 2.4	30.7 $\pm$ 2.7	65.6 $\pm$ 1.9
Leupeptin	10.7 $\pm$ 1.2 $\ddagger$	33.9 $\pm$ 3.9 $\ddagger$	22.7 $\pm$ 1.3	45.0 $\pm$ 3.4 $\ddagger$	12.9 $\pm$ 3.7	38.9 $\pm$ 4.4	28.5 $\pm$ 1.8	59.6 $\pm$ 1.5
Methylamine	7.5 $\pm$ 0.8*	25.9 $\pm$ 2.7*	22.5 $\pm$ 4.6	40.4 $\pm$ 4.3 $\ddagger$	12.6 $\pm$ 1.2	37.1 $\pm$ 1.4	27.5 $\pm$ 3.1	58.4 $\pm$ 5.2

various reagents after incubations of 5 and 24 h in basal and step-down medium. Only methylamine caused an increase in trichloroacetic acid-precipitable radioactivity in the cell medium from 9 to 15% of medium radioactivity. Methylamine also slightly increased lactate dehydrogenase release from cells, indicating that the increase in trichloroacetic acid-precipitable radioactivity was due to cell breakage as opposed to increased secretion or cell detachment from the monolayer. For normal long-labelled proteins, inhibition of degradation by methylamine was greater in step-down medium than in growth medium. The effects of methylamine were less pronounced in normal short-labelled proteins and abnormal proteins of long and short labelling periods. These results indicated that the degradation of protein can be put in order of decreasing lysosomal involvement as: normal long-labelled proteins > normal short-labelled proteins > abnormal long-labelled proteins > abnormal short-labelled proteins.

Leupeptin showed results similar to those produced by methylamine, but in most cases the extent of the inhibition of the protein degradation was smaller.

The results obtained with colchicine were not expected. In step-down conditions, colchicine had a negligible effect upon protein degradation, whereas in serum-containing medium, although effects upon short-labelled normal protein and both classes of abnormal proteins were slight, colchicine at 25 μ M stimulated degradation of normal long-labelled proteins in every experiment, and a two-tailed *t* test showed that protein degradation was statistically significantly raised from 29.6 $\pm$ 1.0% ($n = 13$) to 34.5 $\pm$ 2.0% ($n = 11$) ($P < 0.0001$) after 24 h incu-

Table 3. *Percentage inhibition of basal protein degradation by combinations of colchicine, vinblastine and methylamine*

Results are the means of three determinations. Concentrations and conditions were as for Table 2. Results are $\pm$ S.D.

Reagent	Time (h)	Inhibition (%)	
		5	24
Colchicine		-12.7 $\pm$ 3.5	-16.6 $\pm$ 3.3
Vinblastine		-8.3 $\pm$ 3.6	-17.9 $\pm$ 0.4
Colchicine + methylamine		12.7 $\pm$ 4.8	13.7 $\pm$ 2.8
Vinblastine + methylamine		11.7 $\pm$ 1.6	11.6 $\pm$ 2.6
Methylamine		23.0 $\pm$ 2.6	30.5 $\pm$ 1.4

bation. As these results were at variance with those found in other types of cells, we decided to investigate this phenomenon further, by using an alternative microtubule inhibitor, vinblastine sulphate, and combinations of inhibitors. The results are shown in Table 3. These show that the results for vinblastine closely resemble those for colchicine, indicating that inhibition of microtubule function, and not another property of colchicine, is causing this increase in degradation. Also, it was found that the effects of colchicine or vinblastine sulphate and methylamine are additive, indicating that the increase in degradation was not lysosomal in origin.

Addition of microtubule inhibitors to cells incubated in growth or step-down conditions in the presence of methylamine did not affect the degree of vacuolation of cells (S. A. Wharton & P. A. Riley, unpublished work), indicating that the effects of methylamine are not blocked by microtubule inhibi-

tors. Experiments measuring the uptake of [*methyl*-³H]methylamine showed that microtubule inhibitors had little effect upon methylamine uptake. This uptake was found to be 40.4 c.p.m./24 h per μg of cell protein in growth medium and 37.1 c.p.m./24 h per μg of cell protein in step-down medium. Colchicine (25 μM final concn.) and vinblastine (10 μM) decreased these amounts by 10.4 and 13.6% in growth medium and by 8.4 and 19.7% in step-down medium respectively. From the specific radioactivity of the methylamine (0.36 $\mu\text{Ci}/\mu\text{mol}$) and the protein content per cell ($1\mu\text{g}/4.4 \times 10^3$ cells) it can be calculated that, in the control incubation, approx. 2×10^{10} methylamine molecules per cell were taken up after 24 h, which is twice as high as can be accounted for by passive diffusion into the cell (assuming a cell volume of $3000\mu\text{m}^3$), indicating that accumulation of methylamine into cells has occurred. The above experiments therefore indicate that the stimulation in protein degradation caused by the microtubule inhibitors is via a pathway not inhibited by methylamine.

Discussion

The present results have all been obtained by using cells at the same state of confluence in culture conditions that have been rigidly controlled. Free radiolabelled amino acids were washed from the cells before degradation studies, and re-utilization was kept minimal.

A desirable feature of protein-degradation studies is the use of a radiolabelled amino acid that is not significantly metabolized by the cells in case the metabolites are re-incorporated into the cells. Although it has been reported that this amino acid is metabolized by rat diaphragm muscle (Fulks *et al.*, 1979), valine was not measurably metabolized by the MRC5 cells and thus is very suitable for protein-degradation studies. Experiments on protein degradation with arginine are valid (shown by the similar results for L-[3,4-³H]valine and L-[guanido-¹⁴C]arginine-containing proteins); however, experiments measuring the absolute amounts of incorporation of arginine into proteins might not be. An arginase activity was demonstrated in foetal-calf serum by Kihara & De la Flor (1968), who also found it to be heat-stable. This arginase activity might become important in long-term tissue culture experiments, as it would deplete the cells of an essential amino acid. Negligible arginase activity was found in the cells, so that, as the cells did not take up large amounts of radiolabelled urea from the medium, re-incorporation of label into cellular components would be minimal. As the arginine or canavanine is only metabolized in the culture medium, and the resulting radiolabelled product was trichloroacetic acid-soluble, the arginase activity will

not affect protein-degradation determination. Although no reports of arginase activity in foetal bovine lung fibroblasts could be found, Herzfeld & Raper (1976) reported that lung fibroblasts exhibit negligible activity compared with liver cells, and foetal liver had much less activity than the adult liver. The ability of this arginase activity to metabolize canavanine to urea and canaline is another example of the similarity in metabolism of canavanine and arginine, which has been reviewed by Rosenthal (1977). Competition between the two compounds is shown by the lower conversion of canavanine into arginine-rich medium compared with arginine-free labelling medium. Canavanine has been shown to be incorporated into the protein of cells at the expense of arginine by fibroblasts (Schein *et al.*, 1980) and HeLa cells (Prouty, 1976), and, as the MRC5 only incorporated radiolabel into a macromolecule that could be acid-hydrolysed to give free canavanine, this seems to be the case in this cell type.

Knowles & Ballard (1976) reported that canavanine-containing proteins were degraded five times faster than normal proteins. However, they compared normal proteins labelled for 16 h with canavanine-containing protein labelled for 3 h, and since long labelling periods preferentially label stable proteins (Poole & Wibo, 1973), these results are biased for the more rapid degradation of the abnormal protein. We have compared degradation of proteins labelled for the same length of time to prevent this bias. The results showed that the rates of degradation of labelled abnormal proteins could be manipulated in very much the same way as normal proteins by varying the period of labelling. However, the degradation of abnormal protein was always greater than that of normal proteins labelled for the same time and, interestingly, the percentage increase in degradation so brought about was the same for both long and short labelling periods.

Long-labelled proteins exhibited a greater increase of degradation in step-down medium and more lysosomal involvement in basal degradation than for short-labelled proteins, in agreement with previous studies (Dean, 1980; Amenta & Brocher, 1981; Seglen *et al.*, 1979; Neff *et al.*, 1979). Comparison of long- and short-labelled abnormal proteins showed a similar effect, although this was less pronounced because in both cases lysosomal involvement in degradation was much less than for the corresponding normal proteins. The most interesting comparison is to be made between abnormal long-label proteins and normal short-label proteins. Although the former are degraded more slowly (25% of protein is degraded in 12 h as opposed to 6 h for the abnormal proteins), the lysosomal involvement and increase in step-down conditions is greater in the normal proteins. Therefore the degree of

lysosomal involvement in basal degradation is not a simple function of the half-lives of proteins studied, since abnormal proteins seem to be preferentially degraded non-lysosomally. Other studies have shown that abnormal proteins are degraded non-lysosomally (Knowles & Ballard, 1976; Neff *et al.*, 1979), but in both reports the abnormal proteins were the fastest-turning-over protein group studied, and an interpretation of these results could be that the more labile proteins are degraded by cytosolic proteinases before they can gain access to the lysosomal proteinases by autophagy, so that the degree of lysosomal involvement is inversely proportional to the half-lives of the proteins. Our results show that this is not the case; either there is a cytosolic proteinase specific for abnormal proteins which, perhaps, spontaneously denature and are thus recognized as substrates, as has been reported in reticulocytes (Etlinger & Goldberg, 1977) and rat liver (De Martino & Goldberg, 1979), or that abnormal proteins are excluded from the lysosomal compartment and so are degraded almost exclusively in the cytosol.

Use of colchicine, leupeptin or methylamine was found not to impair cell viability and thus comparison between these effects on protein degradation are valid. Leupeptin showed a similar pattern of inhibition to methylamine, except that inhibition was less. Leupeptin is an inhibitor of thiol proteinases (Barrett, 1980), therefore this reagent is likely to have less effect upon lysosomes than does methylamine, as these organelles contain many other carboxy and serine proteinases. The fact that when most protein degradation is cytosolic in nature, such as with abnormal proteins, the inhibition by leupeptin is not increased indicates that the cytosolic Ca^{2+} -activated thiol proteinase does not degrade these proteins to a significant extent.

Colchicine caused the most unexpected results in that long-labelled basal degradation was stimulated 16% in a statistically significant manner ($P < 0.0001$), although this reagent had no effect upon step-down degradation. Vinblastine also increased basal degradation. All other experiments with different cells *in vitro* have shown that colchicine had no effect upon basal degradation, but inhibited step-down degradation (e.g. Amenta *et al.*, 1977). It has been reported that vinblastine and colchicine stimulate the formation of autophagic vacuoles *in vivo* in rat liver (Marzella & Glaumann, 1980; Marzella *et al.*, 1980) and mouse liver, which is associated with an increase in proteolysis. This increase was postulated to involve the lysosomal pathway of degradation because it was inhibited by NH_4^+ , cycloheximide and insulin. We found that the increase in degradation was non-lysosomal in character. Marzella & Glaumann (1980) reported that ammonium ions at 10 mM only inhibited

degradation by 26%, whereas vinblastine stimulated proteolysis severalfold; it seems, therefore, that not all their increase was due to an activation of lysosomal proteinases and that other pathways are involved. It has been reported that colchicine and vinblastine cause a disruption of the Golgi and a change in the distribution of lysosomes (Piasek & Thyberg, 1980; Thyberg *et al.*, 1980), and these morphological changes could affect protein degradation. Alternatively, the leakages of lysosomal contents caused by microtubule inhibitors (Marzella & Glaumann, 1980) could explain the increase in protein degradation, as lysosomes contain enzymes still active at neutral pH values (Kirschke *et al.*, 1977) that would not be affected by methylamine. On the other hand, colchicine could stimulate cytoplasmic proteolytic activity in a similar manner to that reported to occur in platelets (Wittels *et al.*, 1981). Further experiments would have to be carried out to see which, if any, of the above processes applies.

The increase in degradation in the presence of colchicine might have a physiological function, since, as microtubule inhibitors trap cells in mitosis (Dustin, 1978), proteolysis might be increased to prevent an accumulation of cellular protein. It has been shown that when hamster fibroblasts are brought to quiescence by high culture density, degradation of endogenous protein is increased (Hendil, 1980).

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