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Inhibition of Protein Degradation in Isolated Rat Hepatocytes

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1. Isolated parenchymal cells were prepared by collagenase perfusion of livers from fed rats that had been previously injected with [^{3}H]leucine to label liver proteins. When these cells were incubated in a salts medium containing glucose, gelatin and EDTA, cellular integrity was maintained over a period of 6h. 2. Cells incubated in the presence of 2mM-leucine to minimize radioactive isotope reincorporation released [^{3}H]leucine into the medium at a rate accounting for the degradation of 4.5% of the labelled cell protein per h. 3. Degradation of [^{3}H]protein in these cells was inhibited by insulin and by certain amino acids, of which tryptophan and phenylalanine were the most effective. 4. Protein degradation was decreased by several proteinase inhibitors, particularly those that are known to inhibit lysosomal cathepsin B, and by inhibitors of cell-energy production. 5. Ammonia inhibited degradation, but only at concentrations above 1.8mM. Aliphatic analogues of ammonia were effective at lower concentrations than was ammonia. 6. High concentrations of ammonia inhibited degradation by 50%. The extent of this inhibition could not be increased further by the addition of the cathepsin B inhibitor leupeptin, which by itself inhibited degradation by approx. 30%. 7. The sensitivity of proteolysis in isolated hepatocytes to these various inhibitory agents is discussed in relation to their possible modes of action.

Previous attempts to elucidate the control of intracellular protein degradation in liver have utilized the isolated perfused organ. Isolation of the liver for such studies has eliminated inter-organ influences and permitted the addition of effectors. Demonstration of an inhibition by insulin (Mortimore & Mondon, 1970) and amino acids (Woodside & Mortimore, 1972) as well as a stimulation by glucagon (Miller, 1960; Mallette *et al.*, 1969; Woodside *et al.*, 1974) of protein degradation has been possible, despite the need for relatively long perfusion experiments.

The purpose of the present study was to further examine factors that might regulate protein degradation in liver. Rat hepatocyte suspensions were chosen as the experimental system, because many replicate tests could be conducted on each liver, and in addition the possibility of a variable contribution by non-parenchymal cells could be eliminated. Since the average rates of protein degradation *in vivo* are relatively slow (2–3%/h; Garlick *et al.*, 1973), it was necessary to develop incubation conditions suitable for the maintenance of liver cells for several hours.

Materials and Methods

Chemicals

L-[4,5-^{3}H]Leucine (specific radioactivity 50–60Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; collagenase (CLS-45 type 1) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; streptomycin sulphate and penicillin G were from Glaxo–Allenburys Ltd., Boronia, Vic., Australia; gelatin was from Davis Gelatine (Australia) Pty. Ltd., Botany, N.S.W., Australia; insulin (sterile solution, 40units/ml) was Actrapid from Novo Industri A/S, Copenhagen, Denmark; leupeptin, a pepstatin, antipain and bestatin were kindly provided by Dr. H. Umezawa, Microbial Chemistry Research Foundation, 14–23, Kamiosaki 3-Chome, Shinagawa-Ku, Tokyo, Japan; NCS solubilizer was from Amersham/Searle Corp., Arlington Heights, IL, U.S.A.; methylamine hydrochloride, ethylamine and ethanolamine were from E. Merck A.G., Darmstadt, W. Germany; [*carboxy*-^{14}C]inulin was from New England Nuclear Corp., Boston, MA, U.S.A.; and cycloheximide, puromycin, Tos-Lys-CH_2Cl (7-amino-1-chloro-3-tosylamidoheptan-2-one; TLCK), Tos-Phe-CH_2Cl (1-chloro-4-phenyl-3-tosylamidobutan-2-one; TPCK), phenylmethanesulphonyl fluoride, jack-bean urease (type IV), Tes,‡ Trypan Blue, EGTA and all amino acids were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Amino acid mixtures

The mixture of essential amino acids based on that of Eagle (1959) was used with components in the

‡ Abbreviation: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

following proportions: Arg, 10; His, 2.5; Lys, 6.4; Leu, 8; Ile, 8; Met, 2; Phe, 4; Thr, 8; Try, 1; Val, 8; Tyr, 4; Cys, 2. The mixture of non-essential amino acids contained Ala, Asn, Asp, Glu, Pro, Ser and Gly in equimolar proportions.

Animals

Male rats were fed *ad libitum* on a high-carbohydrate diet containing 20% protein and 4% fat. Fed rats (135–150g) were injected intraperitoneally with 250μCi of [^{3}H]leucine 1.5h before removal of the liver; during this time the rats were kept in the dark with free access to food.

Preparation and incubation of hepatocytes

Liver parenchymal cells were isolated by the technique of Berry & Friend (1969), except that hyaluronidase was omitted and the final concentration of collagenase in the perfusate (Krebs improved Ringer I with pyruvate; Dawson *et al.*, 1959) was 0.037%. The buffer used for incubations consisted of Ca^{2+}-free Krebs–Henseleit saline (Dawson *et al.*, 1959) plus 2mM-leucine, 16.5mM-glucose, 20mM-Tes, 100μM-EDTA and 1.5g of gelatin, 1mg of Phenol Red, 6mg of penicillin and 10mg of streptomycin sulphate per 100ml. The gas phase for the incubations and against which the buffer was equilibrated was O_2/CO_2 (19:1). The pH was 7.4 at 37°C.

The incubations were conducted in polyethylene scintillation vials (20ml capacity) each containing 2×10^6–3×10^6 cells in 1.275ml of buffer. Additions were made in a volume of 0.225ml, and NaCl was added so that the final osmolarity was 300mosM. Vials were shaken at 90 oscillations/min under an atmosphere of O_2/CO_2 (19:1) at 37°C.

Measurement of protein degradation

At the beginning and end of the incubation period, 1ml samples were centrifuged at 50g for 2min to separate cells from medium. $HClO_4$ was added to each fraction to give a final concentration of 0.85M. Samples of the $HClO_4$-soluble (amino acids) and $HClO_4$-insoluble (protein) fractions were dissolved in NCS solubilizer, added to toluene scintillation fluid and radioactivity was determined in a Packard 3375 scintillation spectrometer (Hopgood *et al.*, 1973).

During incubation both ^{3}H-labelled amino acids and [^{3}H]protein are released into the medium (Fig. 1). Control experiments have shown that 95% of the radioactivity of the amino acid fraction was present as leucine, that the [^{3}H]protein contains a broad molecular-weight spectrum of proteins similar to liver supernatant protein, and that this [^{3}H]protein released into the medium is not subsequently hydrolysed to radioactive amino acids. The extent of the release of [^{3}H]protein from the cells is expressed as a percentage of the total [^{3}H]protein:

$$\%\ \text{protein release} = \frac{[^3\text{H}]\text{protein in medium}}{[^3\text{H}]\text{protein in cells plus medium}} \times 100$$

Since [^{3}H]protein release occurs at an approximately linear rate with time, one-half of the released protein radioactivity is not assessed as a potential substrate for intracellular degradation. Thus:

$$\%\ \text{degradation of protein} = \frac{^3\text{H-labelled amino acids in cells plus medium}}{\text{total radioactivity} - \left(\dfrac{[^3\text{H}]\text{protein in medium}}{2}\right)} \times 100$$

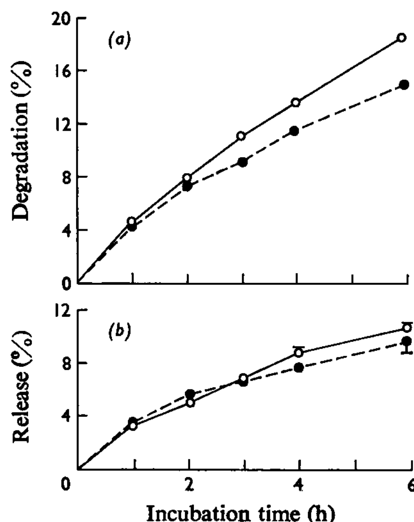

Fig. 1. *Time-course of [^{3}H]protein degradation (a) an release (b)*

Hepatocytes were prepared and incubated, [^{3}H]-protein degradation and release measured, and results expressed as described in the Materials and Methods section. Cells were incubated without additions (○) or in the presence of 7nM-insulin plus the essential amino acid mixture given in Table 5 (●). Values are means for three determinations at each time period. The S.E.M. is indicated when it is sufficiently large.

Measurements of cellular integrity

Lactate dehydrogenase (EC 1.1.1.27) activity released into the medium was assayed directly (Kornberg, 1955). Total activity in cells plus medium was measured in a cell suspension diluted 10–20-fold in the presence of 0.5% Triton X-100.

The proportion of non-viable cells was estimated by mixing the cell suspension with 2vol. of freshly prepared Trypan Blue (0.2% in incubation buffer), after which a minimum of 100 cells were examined for Trypan Blue uptake.

Intracellular K^+ content of the cells was measured after separation of the cells from the medium by the method of Hems *et al.* (1975), modified in that the neck of the centrifuge tube was filled with 0.2M-sucrose, and the bowl contained 0.7M-$HClO_4$ and 0.16M-sucrose. The tubes were centrifuged at 3000*g* for 1 min, and the K^+ concentration in the bowl solution was measured by flame photometry. To correct for medium carried down with the cells, [^{14}C]inulin was added to cell samples before centrifugation and the radioactivity determined in the bowl solution by liquid-scintillation spectrometry (Hopgood *et al.*, 1973).

Metabolite assays

Protein was precipitated from the incubation medium by the addition of $HClO_4$ to a concentration of 0.85M. After centrifugation at 3000*g* for 5min, 3M-$KHCO_3$ was added to the supernatant to give a final pH of 7 and the $KClO_4$ removed by a second centrifugation. Ammonia was measured with glutamate dehydrogenase (EC 1.4.1.2) in portions of this neutralized extract (Kun & Kearney, 1974). Subsequently urease (50μg) was added to the same cuvette to measure ammonia liberated from urea.

Results and Discussion

Incubation conditions

In preliminary experiments cells were incubated in the absence of EDTA. Under these conditions the amount of intracellular [^{3}H]protein released was approx. 5%/h, suggesting poor viability of the cells. Protein release was greatly decreased by the addition of a mixture of essential amino acids (Table 1). Methionine, which was present in the mixture at 0.5mM, was particularly effective at decreasing protein release. Some inhibition was found at methionine concentrations as low as 50μM, and a maximal effect occurred at 200μM. Although these observations could be explained by an amino acid deficiency in the cell preparations, it was observed that EDTA at a concentration of 50μM was even more effective than either the mixture of essential amino acids or methionine in decreasing protein release (Table 1). Perhaps all these effects are exerted by a decrease in the concentration of metal ions, particularly since methionine is known to chelate bivalent metal ions (Martell & Calvin, 1952). Further, the effect of EDTA is not modified by the addition of the mixture of essential amino acids, suggesting that the inhibition of protein release is exerted by the same mechanism. The effect of EDTA is apparently not related to binding of Ca^{2+}, as the more specific Ca^{2+}-chelator, EGTA, is markedly less effective than EDTA at decreasing protein release (Table 1).

Further parameters of cellular integrity were examined in cells incubated in the presence and absence of EDTA (Table 2). Cells incubated for 4h in the absence of EDTA released into the medium approximately one-third of the initial lactate dehydrogenase activity and one-quarter of the [^{3}H]protein. Almost half of these same cells showed uptake of Trypan Blue. As reported by Baur *et al.* (1975), the most sensitive index of cellular damage was the intracellular K^+ concentration, which decreased to 24mM in the absence of EDTA. In the presence of 100μM-EDTA a substantial decrease was found in all indices of cellular damage, indicating that a large proportion of the cells remained intact throughout the 4h incubation period. On this basis 100μM-EDTA was included in the medium for all subsequent experiments.

The time-course of the degradation and release of intracellular [^{3}H]protein during incubation is shown in Fig. 1. Approx. 20% of the protein was converted

Table 1. *Effects of amino acids and metal chelators on protein release*

Details of the preparation and incubation of hepatocytes are given in the Materials and Methods section, except that EDTA was omitted from the incubation buffer. The essential amino acid mixture was at a total concentration of 16mM in the proportions given in the Materials and Methods section. Incubation time was 4h. Values are means ± S.E.M. for the numbers of determinations given in parentheses.

Additions	Percentage of [^{3}H]protein released
None	26.9 ± 2.0 (15)
Essential amino acids	12.1 ± 0.4 (15)
Methionine: 20μM	20.8 ± 3.6 (4)
50μM	18.3 ± 1.4 (4)
200μM	14.4 ± 0.7 (4)
2mM	14.4 ± 0.7 (5)
EDTA: 10μM	24.1 ± 1.2 (4)
50μM	7.9 ± 0.2 (4)
500μM	7.0 ± 0.5 (4)
Essential amino acids + 50μM-EDTA	7.2 ± 0.5 (4)
EGTA: 100μM	19.3 ± 0.2 (4)

Table 2. *Effects of EDTA on hepatocyte integrity*

Experimental details are given in Table 1 and assay procedures in the Materials and Methods section. Data are means of individual values from two separate experiments.

Measurement	EDTA (μM)	Incubation period 0h	Incubation period 4h
[^{3}H]Protein release (%)	0	2.4	23.5
	100	2.2	11.6
Trypan Blue staining (%)	0	12	45
	100	11	15
Lactate dehydrogenase release (%)	0	3.3	33
	100	3.2	13
Cell K$^+$ (mM)	0	86	24
	100	86	91

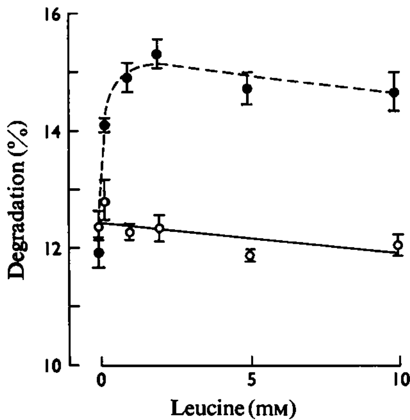

Fig. 2. *Effect of leucine concentration on degradation in the presence and absence of cycloheximide*

Details are as for Fig. 1 except that leucine concentration in the incubation buffer was varied as shown. The incubation time was 4h. Cells were incubated with no additions (●) or with 3.4μM-cycloheximide (○).

into amino acids in 6h. The rate declined from 4.5%/h in the first hour to 2.5%/h in the final 2h. Protein release from the cells showed a similar trend with time, but the amount of [^{3}H]protein lost from the cells was substantially less than the amount degraded. Fig. 1 also shows that the addition of amino acids plus insulin resulted in an inhibition of degradation throughout the incubation period, but had only a marginal effect on protein release.

Reincorporation into protein of [^{3}H]leucine released by protein degradation results in an underestimation of the measured accumulation of radioactivity in the amino acid fraction. This effect was assessed by varying the leucine concentration in the incubation medium (Fig. 2). The extent of measured degradation was increased dramatically by the addition of 0.2mM-leucine, and a maximum increase was obtained at 1mM-leucine. At higher leucine concentrations, degradation was slightly decreased, possibly owing to a small direct inhibition of degradation by the amino acid.

Table 3. *Inhibition of protein degradation by protein-synthesis inhibitors*

Hepatocytes were prepared and incubated as described in the Materials and Methods section and incubated for 4h. Inhibition of degradation by added agents is expressed as a percentage of the total degradation observed with no additions. Values are means ± S.E.M., for the numbers of determinations in parentheses.

Additions	Percentage inhibition of degradation
Puromycin: 30μM	13.0±1.3 (4)
300μM	19.3±2.5 (4)
Cycloheximide: 0.34μM	9.3±1.9 (4)
3.4μM	20.4±1.5 (7)
Insulin (7nM)	3.6±0.9 (8)
Insulin (7nM)+cycloheximide (3.4μM)	23.3±1.7 (8)
Essential amino acids+insulin (7nM)	22.9±1.6 (8)
Essential amino acids+insulin (7nM)+ cycloheximide (3.4μM)	35.4±2.1 (8)

Changes in protein synthesis caused by added compounds may also affect leucine reincorporation, making interpretation of effects on protein degradation equivocal. We have tested this proposal by adding cycloheximide to the incubation medium at a concentration known to inhibit protein synthesis in hepatocytes by 95% (Schreiber & Schreiber, 1973). However, the increase in apparent degradation caused by the leucine chase was not found when cycloheximide was present (Fig. 2). This effect is opposite to that predicted if cycloheximide was acting solely to prevent reincorporation. We suggest that cycloheximide has two effects; one causing an apparent increase in degradation by preventing reincorporation, and a second, over-riding, effect producing an inhibition of protein degradation. Accordingly, cycloheximide is inappropriate for these studies, and we have used the leucine chase method to minimize reincorporation in all subsequent experiments.

The effects of protein-synthesis inhibitors on protein degradation was examined in further experiments with cycloheximide and puromycin. Table 3 shows that both compounds are inhibitors of protein degradation and that almost complete inhibition of protein synthesis by either of these agents (Schreiber & Schreiber, 1973) leads to a 20% inhibition of degradation. The inhibition by 3.4μM-cycloheximide

Table 4. *Inhibition of degradation by inhibitors of energy production*

Experimental details are as given in Table 3.

Additions	Percentage inhibition of degradation	Percentage of [³H]protein released
None	—	10.6±0.4 (6)
NaF: 1 mM	9.4±1.5 (3)	11.0±0.3 (3)
10 mM	61.0±0.6 (6)	20.1±1.8 (6)
NaN_3: 1 mM	18.6±0.9 (3)	11.2±0.1 (3)
10 mM	64.0±1.4 (6)	34.3±0.6 (6)
2,4-Dinitrophenol: 0.1 mM	6.2±1.2 (3)	11.9±0.3 (3)
0.3 mM	55.8±4.7 (6)	22.2±3.0 (6)
Iodoacetate: 0.05 mM	9.5±1.4 (3)	10.3±0.6 (3)
0.5 mM	75.6±0.7 (6)	35.4±0.7 (6)
Rotenone: 0.025 mM	36.3±4.4 (6)	10.1±0.6 (6)
0.25 mM	58.8±0.9 (3)	17.8±0.8 (3)
Phenazine methosulphate:		
0.05 mM	68.5±0.7 (3)	9.1±0.2 (3)
0.25 mM	82.8±1.3 (6)	22.7±0.7 (6)

is additional to that produced by insulin alone or by insulin in the presence of amino acids. In the latter case, the total inhibition of degradation was 35%. Although an inhibition of protein degradation by cycloheximide and other protein-synthesis inhibitors has been noted with several cell types (Goldberg & Dice, 1974), the mechanism of the effect is not known. Possibilities include: the inhibition of synthesis of some protein required for protein degradation (Epstein *et al.*, 1975); the accumulation of precursors of proteins such as charged tRNA after inhibition of protein synthesis (Goldberg, 1971), and they in turn decrease protein degradation; and a decrease in the rate of formation of autophagic vacuoles (Neely *et al.*, 1974).

Effects of metabolic inhibitors

It has been observed in many cell types that protein degradation is inhibited by compounds that interfere with energy production (Goldberg & St. John, 1976), probably owing to a requirement for energy for protein degradation. A variety of such inhibitors, including ones acting on glycolysis, oxidative phosphorylation and electron transport, were tested and found to inhibit protein degradation in isolated hepatocytes (Table 4). Fluoride, azide, dinitrophenol and iodoacetate were less effective than rotenone or phenazine methosulphate. At concentrations of the former compounds that significantly inhibited degradation, [³H]protein release was increased, presumably indicating that the cells were unable to maintain cellular integrity in their presence. In contrast, rotenone and particularly phenazine methosulphate inhibited degradation without causing cell breakage, and it should be considered that these compounds inhibit degradation independently of any effect on cellular integrity.

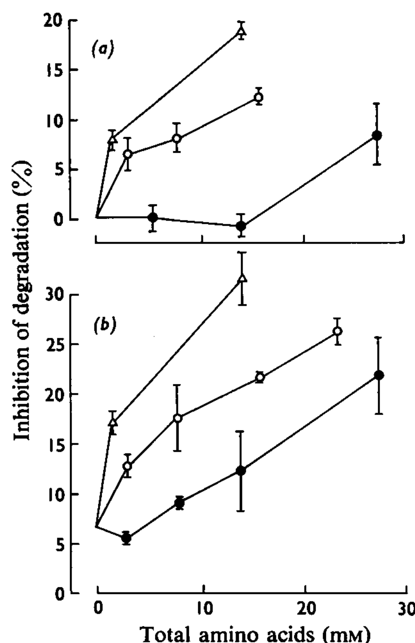

Fig. 3. *Inhibition of protein degradation by amino acid mixtures in the presence and absence of insulin*

Incubation conditions were as given in Fig. 1 with an incubation period of 4h. Inhibition of degradation by amino acids is expressed as a percentage of that observed with no additions. Inhibition was measured (*a*) in the absence and (*b*) in the presence of 7 nM-insulin. The non-essential amino acids mixture (●) contained Ala, Asn, Asp, Glu, Pro, Ser and Gly in equimolar proportions. The essential amino acids mixture (○) contained amino acids in the molar ratios given in the Materials and Methods section. The Met/Phe/Trp mixture (△) contained the same ratios of these amino acids as in the essential amino acid mixture. Values are means ± S.E.M. for between three and five determinations.

Inhibition by amino acids and amines

A mixture of essential amino acids at total concentrations between 3.2 and 16 mM inhibited degradation in hepatocytes by 6–12%, whereas a mixture of non-essential amino acids was ineffective at similar concentrations (Fig. 3*a*). Insulin alone (7 nM) inhibited degradation by approx. 7% (Fig. 3*b*), whereas the addition of either amino acid mixture caused an inhibition that was approximately additive to that caused by insulin (Fig. 3*b*).

To determine which amino acids were effective, mixtures of essential amino acids were tested for their

Table 5. *Inhibition of degradation by amino acids in the presence of insulin*

Incubation conditions are described in Table 3. All incubations, except for control, contained insulin. The inhibition of degradation by insulin and by insulin plus various amino acids is expressed as a percentage of the degradation observed in controls. The concentrations of amino acids were the same as present in the essential amino acid mixture (Arg, 2.5mM; His, 0.63mM; Lys, 1.6mM; Leu, 2mM; Ile, 2mM; Met, 0.5mM; Phe, 1mM; Thr, 2mM; Trp, 0.25mM; Val, 2mM; Tyr, 1mM; Cys, 0.5mM), except where otherwise indicated. Values are means ± S.E.M. for the numbers of determinations in parentheses.

Additions	Percentage inhibition of degradation compared with minus-insulin controls
None	7.5 ± 1.8 (3)
Essential amino acids	21.2 ± 1.9 (6)
Arg, His, Lys, Thr, Cys, Met, Phe, Trp	18.2 ± 1.0 (6)
Leu, Ile, Val, Tyr	10.7 ± 1.3 (6)
Arg, His, Lys, Thr, Cys, Leu, Ile, Val, Tyr	13.6 ± 1.4 (6)
Met, Phe, Trp	17.0 ± 1.2 (10)
Met (4mM), Phe (8mM), Trp (2mM)	31.5 ± 2.7 (10)
Met (4mM)	10.4 ± 1.3 (3)
Phe (8mM)	17.3 ± 0.9 (3)
Trp (2mM)	19.0 ± 1.7 (6)

effects on degradation in the presence of insulin (Table 5). A combination of methionine, phenylalanine and tryptophan inhibited degradation almost as well as the complete mixture of essential amino acids, in spite of the fact that their combined concentrations were only one-tenth that of the total. The inhibition by these three amino acids, when present at a total concentration similar to that of the complete mixture (14mM), was greater than that found with the complete mixture (Table 5). When tested individually at the higher concentrations, none of the amino acids retained the full inhibitory activity, although phenylalanine and tryptophan were more effective than methionine (Table 5). The inhibitory effect of the methionine, phenylalanine and tryptophan mixture was also observed in the absence of insulin (Fig. 3*a*).

We cannot offer any explanation for the selective inhibition by methionine, phenylalanine and tryptophan of protein degradation except to point out that these amino acids often restrict protein synthesis *in vivo* (Munro, 1970). Further, tryptophan alone has been shown to inhibit the degradation of several hepatic enzymes (Schimke *et al.*, 1965; Ballard & Hopgood, 1973).

Inhibition of proteolysis by amino acids has been reported in perfused liver (Woodside & Mortimore, 1972), but could not be shown by Seglen (1975) in isolated hepatocytes. However, nitrogen loss rather than protein degradation itself was measured in the latter study, so that a strict comparison cannot be made.

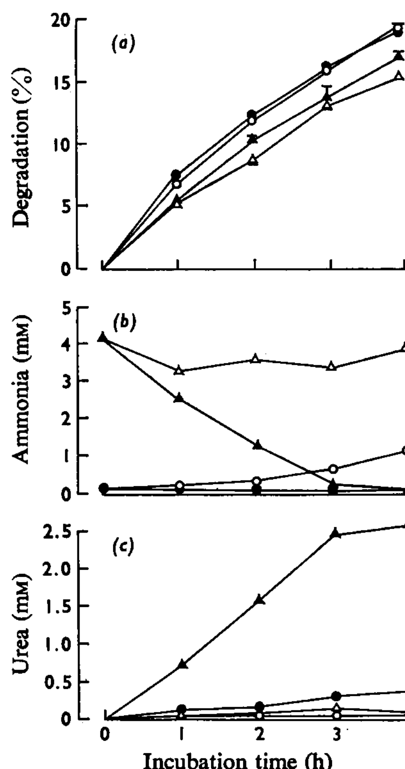

Fig. 4. *Effect of ammonia conversion into urea on inhibition of protein degradation*

Incubation conditions are given in Fig. 1. At the times indicated, samples of cell suspension were centrifuged at 50*g* for 2min, and (*a*) radioactivities in $HClO_4$-soluble and $HClO_4$-insoluble material from cells and medium were measured for determination of percentage degradation; (*b*) ammonia and (*c*) urea were measured in the cell-free medium as described in the Materials and Methods section. Cells were incubated with no additions (●), urease (10μg/ml) (○), 5mM-NH_4Cl (▲), or urease plus NH_4Cl (△). Values are means ± S.E.M. for three incubations at each time period or means from two incubations at each time period.

We have investigated the effects of end products of amino acid catabolism in an attempt to clarify the mechanism whereby amino acids inhibit proteolysis. Initial experiments with ammonia demonstrated a marked inhibition of hepatocyte proteolysis, with inhibition mostly expressed in the first hour of incubation (Fig. 4*a*). Little further

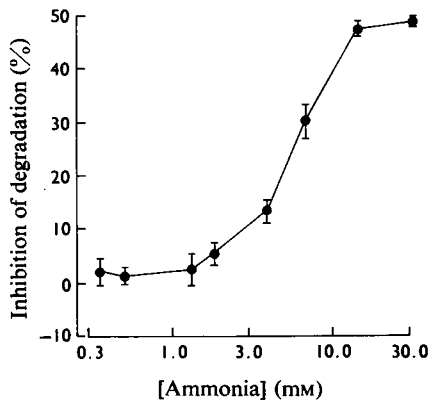

Fig. 5. *Inhibition of degradation by ammonia in the presence of urease*

Incubation conditions are given in Fig. 1. Control incubations contained no added ammonia or urease. All other incubations contained urease (10μg/ml) and various concentrations of NH_4Cl. Ammonia was measured as given in the Materials and Methods section, and concentrations given are the average of those measured at 0 and 4h. Inhibition of degradation is expressed as a percentage of that observed in control incubations. Values are means ± S.E.M. for six determinations.

inhibition occurred during prolonged incubations of the liver cells in the presence of ammonia, an effect explained by the rapid conversion of ammonia into urea in these cells (Williamson *et al.*, 1974). Urea, at concentrations as high as 10mM, did not affect protein-degradation rates. Since accurate measurements of proteolysis require long incubations, we have added urease to the cells at a concentration of 10μg/ml to convert the urea formed into ammonia. Under these conditions the concentration of ammonia was maintained (Fig. 4*b*), accumulation of urea was not found (Fig. 4*c*), and inhibition of proteolysis occurred throughout the incubation period (Fig. 4*a*). It was noted that cells incubated in the absence of ammonia produced a small amount of urea during incubation (Fig. 4*c*), presumably from endogenous substrates, and that the addition of urease to these cells led to the progressive generation of ammonia (Fig. 4*b*). However, the small amount of ammonia formed had an insignificant effect on degradation (Fig. 4*a*).

A concentration curve for the effect of ammonia on protein catabolism in the presence of urease is shown in Fig. 5. The ammonia concentrations given are averaged between the beginning and end of the incubation period in order to allow for endogenous ammonia production. The data in Fig. 5 show that ammonia does not inhibit protein degradation until its concentration exceeds 1.8mM. The inhibition of approx. 50% reached at 13mM-ammonia was not increased by further additions of ammonia. In an attempt to explain the action of ammonia we have investigated the inhibitory properties of several related compounds. Methylamine, ethylamine and ethanolamine inhibit protein catabolism by between 11 and 39% when tested at a concentation of 5mM, with ethylamine being the most effective. Methylamine showed 14% inhibition at 1mM, below the minimum effective concentration noted for ammonia (Fig. 5).

Inhibition of proteolysis by high concentrations of ammonia has also been observed by Seglen (1975), but, since the physiological concentration of this metabolite is probably less than 0.5mM (Williamson & Brosnan, 1974), the physiological significance of the inhibition by ammonia is doubtful. It must be recognized, however, that the local concentration of ammonia at sites of protein degradation may be considerably different from the average cell value. Further, the high rate of urea synthesis would suggest that much lower ammonia concentrations exist intracellularly than in the cells plus medium. Thus the minimum exogenous concentration of 2mM required for an inhibition of proteolysis may be accompanied by low and physiological concentrations within the hepatocyte.

Seglen (1975) reported an 80% inhibition of proteolysis by ammonia concentrations as low as 8mM. Although the inhibition that we observe is much less, even at 30mM-ammonia, we noted in preliminary experiments that ammonia showed an almost complete inhibition of degradation if cells were incubated in the absence of EDTA. However, such inhibition was explained by irreversible damage to the cells, as indicated by release of labelled protein into the medium.

The mechanism of inhibition of proteolysis by ammonia, methylamine and ethylamine is most satisfactorily explained by the penetration of these compounds into lysosomes and an interference with the lysosomal pH. It is also possible that amines are direct inhibitors of lysosomal proteinases. Whatever the explanation, inhibition by ammonia can be considered as a feedback process which decreases protein catabolism under conditions where ammonia removal is limiting. The greater inhibition by ethylamine and methylamine is probably caused by their stability within the hepatocyte, since they are not rapidly metabolized to urea.

Proteolysis inhibitors

The effects of proteinase inhibitors on protein degradation in these cells are shown in Table 6. At the concentrations tested, the cathepsin B inhibitors leupeptin (Aoyagi *et al.*, 1969) and antipain (Suda *et al.*, 1972) as well as the trypsin inhibitor Tos-Lys-

Table 6. *Inhibition of degradation by proteinase inhibitors*
All details are as given in Table 3.

Additions	Percentage inhibition of degradation
Bestatin (50 μg/ml)	9.4 ± 0.4 (3)
Antipain (50 μg/ml)	27.8 ± 2.1 (3)
Leupeptin (50 μg/ml)	26.9 ± 1.9 (10)
Dimethyl sulphoxide (0.3%)	5.0 ± 2.2 (11)
Pepstatin (50 μg/ml) + dimethyl sulphoxide (0.3%)	25.4 ± 1.1 (3)
Tos-Phe-CH_2Cl (50 μM) + dimethyl sulphoxide (0.3%)	13.7 ± 1.9 (5)
Tos-Lys-CH_2Cl (100 μM) + dimethyl sulphoxide (0.3%)	27.9 ± 0.6 (3)
Phenylmethanesulphonyl fluoride (1 mM) + dimethyl sulphoxide (0.3%)	19.2 ± 1.2 (3)
NH_4Cl (5 mM)	22.2 ± 1.9 (5)
Leupeptin (50 μg/ml) + NH_4Cl (5 mM)	36.9 ± 2.6 (5)
NH_4Cl (14 mM)	51.0 ± 3.9 (5)
Leupeptin (50 μg/ml) + NH_4Cl (14 mM)	53.0 ± 1.8 (5)

CH_2Cl (Shaw, 1967) were moderately effective, and significant inhibition of degradation was observed with the cathepsin D inhibitor pepstatin (Aoyagi *et al.*, 1972), the serine proteinase inhibitor phenylmethanesulphonyl fluoride (Gold, 1967), the chymotrypsin inhibitor Tos-Phe-CH_2Cl (Shaw, 1967) and the leucine aminopeptidase inhibitor bestatin. Higher concentrations of the tosyl derivatives were tested, but they caused extensive cell damage and lysis.

Although some information on the relative importance of different proteinases can be obtained from these measurements of inhibitory action, the results must be interpreted with caution until more is known of the membrane-penetration rates of each compound. Perhaps some inhibitors do not penetrate lysosomes and will only inhibit proteolysis when incorporated during the formation of autophagic vacuoles.

Whereas the inhibitory effects of 5 mM-ammonia and leupeptin were approximately additive (Table 6), the 50% inhibition observed in the presence of 14 mM-ammonia was not significantly increased on addition of leupeptin. Such a result could indicate either that these agents inhibit a single pathway of degradation by 50%, or alternatively that a part of the degradation observed, approximately one-half, is fully inhibited. We have suggested from experiments on Reuber H35 hepatoma cells in culture that two pathways of protein degradation occur in cells, only one of which can be inhibited by hormones, nutrients and protein-synthesis inhibitors (Knowles & Ballard, 1976). Non-proteolytic rate-limiting steps are proposed for the second pathway, so that proteolytic inhibitors would only be effective on this second pathway when the proteinase activity is substantially decreased. The present results are consistent with these concepts and demonstrate a similar range of controls to those acting in the tumour cells.

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