Accelerated Breakdown of Reticulocyte Protein Formed under Conditions of Amino Acid Depletion

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1. Labile protein is formed when rat or rabbit reticulocytes are incubated in medium deficient in individual amino acids, especially histidine, valine or alanine. The fraction of unstable protein is increased to about 35% of the total protein synthesized when the histidinyl-tRNA-charging inhibitor, histidinol, is added to histidine-deficient media. 2. The molecular weights of the labile proteins measured by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence of urea are less than haemoglobin and probably represent prematurely terminated haemoglobin chains. 3. Although protein synthesis is always lower under conditions that produce labile protein, inhibition of protein synthesis by fluoride or cycloheximide does not give an effect similar to amino acid depletion. 4. The synthesis of protein in deficient medium does not alter the degradation rate of pre-existing protein in reticulocytes and is thus unrelated to the stringent response in bacteria. 5. We propose that amino acid-deficient medium leads to a decreased charging of the appropriate tRNA, a concomitant decrease in protein synthesis and the degradation of nascent peptides.

A series of metabolic changes takes place in bacterial or mammalian cells as a result of nutrient starvation. These effects, termed the stringent response or the negative pleiotypic response (Hershko *et al.*, 1971), include inhibition of protein synthesis, RNA synthesis and membrane transport of precursors as well as an acceleration of protein degradation. The lack of an essential amino acid triggers the stringent response in bacteria (Cashel, 1969; Sussman & Gilvarg, 1969; Goldberg, 1971), whereas serum starvation or the absence of insulin produce comparable effects in mammalian cells (Hershko *et al.*, 1971; Morhenn *et al.*, 1974). Part of the pleiotypic response can be produced in mouse fibroblasts by amino acid starvation, but protein degradation is not affected (Morhenn *et al.*, 1974). The changes are possibly caused by the accumulation of uncharged tRNA because addition of the tRNA^{His}-charging inhibitor, histidinol, mimics the effects of histidine-deficient medium in that protein synthesis, RNA synthesis and the uptake of low-molecular-weight compounds are decreased (Grummt & Grummt, 1976).

We have investigated protein degradation in reticulocytes starved of individual amino acids and confirm the conclusion of Morhenn *et al.* (1974) that the degradation rate of pre-existing protein is not altered. However, proteins actually synthesized during the amino acid imbalance were found to be unstable, probably as a result of the formation and degradation of incomplete peptides.

Materials and Methods

Chemicals

L-Amino acids, L-O-methylthreonine and L-histidinol were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. L-[4,5-³H]Leucine (specific radioactivity 50–60 Ci/mmol) and L-[U-¹⁴C]leucine (specific radioactivity 300–350 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Phenylhydrazine was crystallized from ethanol as the hydrochloride. Leupeptin and pepstatin were kindly provided by Dr. H. Umezawa, Microbial Chemistry Research Foundation, Shinagawa-Ku, Tokyo, Japan. NCS solubilizer was obtained from Amersham/Searle Corp., Arlington Heights, IL, U.S.A.

Preparation of reticulocytes

Animals were injected subcutaneously with neutralized phenylhydrazine hydrochloride on 5 sequential days and bled on day 7. The daily doses used were 3 mg/100 g for male hooded Wistar rats and 8 mg/kg for male New Zealand White rabbits. Approx. 6 ml of venous blood was obtained in a heparinized syringe from the vena cava of anaesthetized rats or from a rabbit ear vein. The blood was centrifuged at 1000g for 3 min and the cells were washed three times by suspension and centrifugation for 3 min at 1000g and 0°C in a solution containing 145 mM-NaCl, 4 mM-KCl, 1.5 mM-MgCl₂·6H₂O and 2 mM-Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethane-

sulphonic acid) and adjusted to pH 7.5. The washed cells typically consisted of 80–90% reticulocytes as evaluated by staining with Brilliant Cresyl Blue (Gradwohl, 1943).

Labelling of reticulocyte proteins

The reticulocytes were suspended at a concentration equivalent to half that in blood in a solution containing 0.12M-NaCl, 20mM-Tes, 5mM-KCl, 1.5mM-MgCl₂·6H₂O, 5.5mM-glucose, 0.2mM-ferrous ammonium sulphate, 60mg of penicillin/l, 100mg of streptomycin/l, and the following L-amino acids with the one under study being omitted: 0.45mM-arginine, 0.15mM-histidine, 0.6mM-lysine, 0.17mM-isoleucine, 0.15mM-methionine, 0.15mM-phenylalanine, 0.32mM-threonine, 0.1mM-tryptophan, 0.4mM-valine, 0.21mM-tyrosine, 0.1mM-cystine, 0.2mM-cysteine, 1.24mM-alanine, 0.2mM-asparagine, 0.1mM-aspartic acid, 0.15mM-glutamic acid, 1.08mM-proline, 0.75mM-serine, 0.94mM-glycine and 1.19mM-glutamine. The pH was 7.4. After incubation at 37°C for 30min with shaking at 100 oscillations/min to remove endogenous amounts of the amino acid omitted from the medium, reticulocytes from 0.25 ml of blood were pelleted by centrifugation and resuspended in 2 ml of a medium similar to that used for preincubation, but with 6 μ Ci of [³H]leucine and 20nmol of non-radioactive leucine added. Incubation with shaking was carried out for 30min at 37°C. The labelled cells were rapidly washed three times by suspension and centrifugation at 0°C in the solution used for isolation of reticulocytes but with 10 μ M-cycloheximide and 2mM-leucine added to inhibit any reutilization of the [³H]leucine released during proteolysis. Finally the cells were suspended in the same medium used for preincubation, but with 2mM-leucine added, and were used immediately for degradation experiments or for the measurement of protein radioactivity.

Degradation of protein in reticulocytes

Labelled reticulocytes derived from 1 ml of blood were suspended in 2 ml of preincubation medium containing 2mM-leucine and incubated for 2h at 37°C with shaking. At times during this incubation, usually 0, 10, 20, 30, 60 and 120min, 100 μ l portions were removed and mixed with 50 μ l of 30% (w/v) trichloroacetic acid. After centrifugation, the radioactivity in trichloroacetic acid-soluble and trichloroacetic acid-insoluble fractions was determined by liquid-scintillation spectrometry using NCS solubilizer to dissolve the samples. Percentage degradation is taken as 100 times the fraction of total radioactivity in the trichloroacetic acid-soluble fraction.

Deacylation of charged tRNA

Some of the radioactivity in the trichloroacetic acid-insoluble fraction in samples taken immediately

after the labelling period will represent leucyl-tRNA rather than protein. Whereas the fraction of radioactivity in this form will be very small under optimum conditions of protein synthesis, a substantial proportion might be present when protein synthesis is inhibited by amino acid starvation. Accordingly, in some experiments trichloroacetic acid-insoluble fractions were treated to deacylate leucyl-tRNA. In method (1), 200 μ l of 0.5M-glycine adjusted to pH 10 was added to the protein pellet obtained from 100 μ l samples of the degradation mixture, and the solution incubated at 37°C for 1h (Smulson & Thomas, 1969). Protein was precipitated from these samples by the addition of 50 μ l of 100% (w/v) trichloroacetic acid. Soluble and precipitated radioactivity was measured as above. In method (2), 250 μ l of 5 or 10% (w/v) trichloroacetic acid was added to the protein pellet obtained from 100 μ l samples of the degradation mixture and the suspension heated at 90°C for 30min (Siekevitz, 1952). After cooling in ice, the protein was pelleted by centrifugation, and radioactivity in this fraction and in the supernatant was determined as above.

Polyacrylamide-gel electrophoresis of reticulocyte proteins

Reticulocytes were incubated as described under 'Labelling of reticulocyte proteins', except that 50 μ Ci of [³H]leucine was used to increase the isotope incorporation. The cells were washed and degradation was performed as described above in an incubation volume of 2ml. Portions (100 μ l) were taken at zero time and after 120min degradation and added to 900 μ l of dissociation solution to give final concentrations of 1% sodium dodecyl sulphate, 8mM-urea, 1% mercaptoethanol, 10mM-H₃PO₄ and sufficient Tris to bring the pH to 6.8 (Swank & Munkres, 1971). The solution was heated at 60°C for 10min and 100 μ l portions were electrophoresed for 16h at 1.6mA/gel in 12.5% (w/w) acrylamide, 1.25% methylene-bisacrylamide gels (Swank & Munkres, 1971). The gels were fractionated by extrusion, with 40mg fractions being collected and swollen in 5ml of an NCS/ammonia scintillation solution as described by Ward *et al.* (1970). Radioactivity was measured after leaving the vials for 24h with occasional mixing.

Results and Discussion

Synthesis of labile proteins

Protein synthesis in rabbit reticulocytes is inhibited to different degrees when essential amino acids are omitted singly from the incubation medium. The largest effects are noted when histidine and valine are absent (Hori *et al.*, 1967). Our data with rat reticulocytes (Table 1) confirm the earlier experiments and also show that omission of the non-essential amino acid, alanine, also inhibits protein synthesis. The

Table 1. *Effects of amino acid omission on protein synthesis and degradation in rat reticulocytes*

Rat reticulocytes were incubated for 30 min in the presence of [3 H]leucine and all amino acids, except as indicated. Details of protein synthesis and degradation procedures are given in the Materials and Methods section. Values are means $\pm$ S.E.M. for determinations on between four and six preparations of reticulocytes.

Amino acid omitted	Protein synthesis (% of control)	Protein degradation (% in 2h)
None	100	5.5 $\pm$ 1.1
Tryptophan	61 $\pm$ 4	5.2 $\pm$ 1.2
Isoleucine	84 $\pm$ 3	6.1 $\pm$ 1.1
Phenylalanine	69 $\pm$ 6	7.6 $\pm$ 0.9
Lysine	51 $\pm$ 5	9.4 $\pm$ 1.9
Methionine	31 $\pm$ 3	11.2 $\pm$ 1.6
Threonine	40 $\pm$ 7	12.3 $\pm$ 2.4
Alanine	28 $\pm$ 2	14.6 $\pm$ 2.8
Valine	30 $\pm$ 4	15.8 $\pm$ 1.7
Histidine	18 $\pm$ 2	21.5 $\pm$ 2.5
All amino acids	13 $\pm$ 3	17.8 $\pm$ 0.7

relatively large extent of inhibition in the absence of valine, alanine or histidine can be expected as a result of the high proportion of these amino acids in haemoglobin (see Smith, 1975).

When reticulocytes with protein labelled under conditions where one amino acid is omitted from the medium are subsequently incubated for the measurement of protein degradation, an inverse relationship is seen between the inhibition of protein synthesis and the stability of the labelled protein (Table 1). Thus omission of histidine during labelling inhibits synthesis by 82% and gives rise to protein that is degraded at four times the rate of protein synthesized in complete medium. When tryptophan, isoleucine or phenylalanine are omitted from the labelling medium, synthesis is only slightly inhibited and degradation barely affected. Although the results are not presented in Table 1, the omission of aspartate, asparagine, glutamic acid, cystine, arginine, glycine, proline or serine has little effect on synthesis or degradation.

The most likely cause of the inhibition of protein synthesis when an amino acid is limiting is a decrease in the fraction of tRNA acylated. To test whether this hypothesis holds for the accelerated degradation shown in Table 1, we have added the tRNA^{His}-charging inhibitor, histidinol (Hansen *et al.*, 1972), and the tRNA^{Ile}-charging inhibitor, *O*-methylthreonine (Smulson & Rabinovitz, 1968), to the labelling system and subsequently measured protein degradation. The time course of protein degradation in these experiments is shown in Fig. 1. It is apparent that histidinol markedly increases the fraction of labile protein compared with that in the medium lacking histidine, and *O*-methylthreonine also has a

Fig. 1. *Time course of protein degradation after labelling reticulocyte proteins in amino acid-restricted media* Rat reticulocytes were incubated with [3 H]leucine and protein breakdown was measured as described in the Materials and Methods section. Alterations to the labelling medium are: ●, none; ○, isoleucine omitted; △, isoleucine omitted, 10 mM-*O*-methylthreonine added; ■, histidine omitted; ▲, histidine omitted, 3 mM-histidinol added. Protein labelling was inhibited by 19% in the absence of isoleucine, 73% when *O*-methylthreonine was added, 80% in the absence of histidine and 92% when histidinol was added. Comparable results have been found with four other preparations of reticulocytes.

Table 2. *Effect of histidine concentration on the production of labile proteins*

Protein in rat reticulocytes was labelled in the presence of [3 H]leucine and various concentrations of histidine as described in the Materials and Methods section. Histidinol (2 mM) was added during labelling as indicated. Degradation was subsequently measured over a 2 h period in complete medium.

Added histidine (μ M)	Protein degraded in 2h (%)	
	No additions	Histidinol
0	16.2	28.9
1	11.8	26.6
5	9.9	15.6
10	4.7	11.5
25	3.4	6.3
150	3.2	3.6

substantial effect. The inhibitors also decrease the amount of protein labelled below that found in the absence of histidine or isoleucine respectively.

The fraction of labile protein formed is decreased by the addition of very small amounts of histidine to the reticulocyte medium (Table 2) and almost no effect is seen when 10 μ M-histidine is present. Although not shown in Table 2, this concentration of histidine

increases protein synthesis to 72% of that occurring in complete medium. A histidine concentration of $10\mu\text{M}$ is far below the $75\mu\text{M}$ present in the blood of fed rats in the Divisional colony (F. J. Ballard, unpublished work), but slightly higher than values reported in rats 6h after feeding an essentially histidine-free diet ($6\mu\text{M}$; Kumta & Harper, 1962). Since labile protein will only be synthesized in reticulocytes under these extreme conditions, the effect may not be of physiological significance. In the presence of 2mM-histidinol, rather higher histidine concentrations are needed to prevent the synthesis of labile proteins (Table 2), but most of the effect is abolished when $10\mu\text{M}$ -histidine is added together with histidinol. At this concentration protein synthesis has been restored to 38% of the rate found in complete medium.

Nature of the labile protein

In all examples where labile protein has been detected, an inhibition of protein synthesis is noted. Indeed in the various experiments with histidinol protein synthesis in reticulocytes is inhibited by between 92 and 95%. One explanation for the negative correlation between protein labelling and the fraction of unstable protein formed is that protein is not the only substance being measured. An increase-

Fig. 2. Time course of radioactivity in protein, amino acid and deacylated tRNA fractions after labelling reticulocyte protein in the presence of 3 mM-histidinol

Incorporation of [^3H]leucine into histidine-free, histidinol-supplemented medium was measured with rat reticulocytes as described in the Materials and Methods section. After washing, the reticulocytes were incubated in complete medium and 100 μl portions taken for the determination of trichloroacetic acid-soluble (○) radioactivity. The trichloroacetic acid-insoluble fraction was treated with 10% trichloroacetic acid at 90°C for 30 min to deacylate any radioactive leucyl-tRNA present and the radioactivity in the soluble (■) and insoluble (●) fractions was determined.

Table 3. Contribution of deacylation of amino acyl-tRNA to measurements of protein degradation

Degradation of rat reticulocyte protein has been corrected for hydrolysis of radioactive leucyl-tRNA by summing radioactivity in the deacylated trichloroacetic acid-soluble fraction to the radioactivity in the original trichloroacetic acid-soluble fraction before calculating percentage degradation. Values are the average of two experiments. Other details are given in the Materials and Methods section.

Deacylation treatment	Percentage degradation in 2 h of protein labelled in		
	Complete medium	Medium minus histidine	Medium minus histidine plus 3 mM-histidinol
None	2.9	17.6	37.2
5% Trichloroacetic acid, 90°C , 30 min	2.5	15.5	31.2
10% Trichloroacetic acid, 90°C , 30 min	2.7	16.2	30.6
0.5 M-Glycine, pH 10, 37°C , 60 min	2.6	16.9	34.9

ing fraction of the radioactivity in the inhibited states might occur as leucyl-tRNA, since the usual trichloroacetic acid precipitation would include this material with protein. Hydrolysis of leucyl-tRNA during the degradation period by means other than incorporation into protein would produce [^3H]leucine and give an apparent breakdown of protein. We have tested for this possibility by treating the protein fraction with hot 5 or 10% (w/v) trichloroacetic acid or with glycine at pH 10 to deacylate any leucyl-tRNA. Part of the radioactivity in the protein fraction does become acid-soluble after 30 min treatment in 10% trichloroacetic acid at 90°C (Fig. 2). Although the proportion of acid-labile radioactivity in the 'protein' fraction is higher at zero time than at subsequent times during degradation, hydrolysis of leucyl-tRNA can only account for a small part of the measured degradation. This can be seen more readily in Table 3 where the percentage degradation is reported. If instead of calculating percentage degradation in the usual way, namely:

$$\% \text{ degradation} = 100 \times \frac{\text{trichloroacetic acid-soluble radioactivity}}{\text{total radioactivity}}$$

the radioactivity released by deacylation treatment is included in the numerator of the equation, then the true degradation of protein formed in the presence of histidinol is somewhat lower than before. However, the effects with histidinol are smaller when deacyla-

tion is produced by 0.5M-glycine, pH 10, and minimal with any treatment when the degradation of protein synthesized without histidinol is investigated.

Since the labile material formed during histidine starvation is not leucyl-tRNA, it presumably represents some form of modified protein. Such protein could theoretically arise either by premature termination of nascent peptide chains when an uncharged tRNA pairs with the appropriate mRNA codon, or by incorporation of an amino acid other than histidine in response to one or more histidine codons. The first possibility predicts shorter than normal peptide chain lengths and can be readily tested with rabbit reticulocytes where a single type of haemoglobin predominates. Further, haemoglobin normally accounts for almost all of the protein synthesized in rabbit reticulocytes (Yoshikawa & Rapaport, 1974).

Preliminary experiments in which protein labelled in the absence of histidine and presence of histidinol was analysed on Sephadex G-100 columns showed no difference in the molecular-weight spectrum between newly synthesized protein and protein obtained after a 2h degradation period. However, analysis by Sephadex chromatography gives a relatively crude molecular-weight separation, especially since putative shortened haemoglobin peptides might pair with normal subunits to form tetramers barely different in size from native haemoglobin. Accordingly, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with urea added to improve resolution of low-molecular-weight peptides has been used to search for labile peptides of low subunit size (Swank & Munkres, 1971). Staining of such gels with Coomassie Blue gives a single dark band corresponding to the haemoglobin monomer, as well as faint bands at higher molecular weights. The distribution of

radioactivity on gels shows that approx. 30% of proteins have a mobility greater than haemoglobin peptides (Fig. 3a). This radioactivity at lower molecular weights is only found in protein obtained from reticulocytes immediately after labelling in deficient media. With protein from reticulocytes incubated for an additional 2h (Fig. 3b) or from reticulocytes labelled in the presence of a complete medium (results not shown), no radioactivity could be detected with a mobility greater than haemoglobin.

Although the experiment in Fig. 3 indicates the formation of labile peptides with molecular weights less than haemoglobin, it is possible that these are normal complete peptides rather than prematurely terminated haemoglobin chains. Thus inhibition of protein labelling by greater than 90% could selectively inhibit haemoglobin synthesis, emphasizing perhaps a small amount of a protein that does not contain histidine. We think this explanation is unlikely because the omission of any one of many amino acids gives labile protein (Table 1), which would require a number of labile proteins to be present, one lacking histidine, another valine, another alanine etc. Also, all the labile peptides had molecular weights less than haemoglobin (Fig. 1), which is expected if abbreviated haemoglobin chains are being synthesized.

Possible mechanisms for the production of labile protein

Our results showing protein chains with molecular weights less than haemoglobin imply that histidine starvation somehow produces a premature termination during protein synthesis. There are, however, a number of difficulties with this interpretation. For

Fig. 3. Electrophoresis of reticulocyte protein synthesized in the presence of histidine on urea/sodium dodecyl sulphate/polyacrylamide gels

Rabbit reticulocytes were labelled with [^3H]leucine in complete medium without histidine, with 3 mM-histidinol added as described in the Materials and Methods section. Samples were taken immediately after labelling (a) or after 2h incubation of the reticulocytes (b) and electrophoresed as described by Swank & Munkres (1971). Gels were fractionated and counted for radioactivity as described by Ward *et al.* (1970). Marked areas show the position of haemoglobin (1) or insulin (2,3) peptides as detected by staining with Coomassie Blue. Slices have been numbered from the top of the gels.

example, although uncharged tRNA can bind to the unoccupied amino acid site on ribosomes (Moldave, 1965), there is no previous evidence that such binding leads to release of the nascent peptide. Unfortunately we have not been able to test this hypothesis directly because protein labelled in the presence of histidinol, which is labile in whole reticulocytes, is not rapidly degraded in cell extracts even if ATP is added as described by Etlinger & Goldberg (1977). Thus measurement of protein degradation on ribosomes or in ribosome-free supernatants from reticulocytes previously incubated with histidinol are not meaningful.

The effect we have investigated in rat and rabbit reticulocytes may not occur in other cell types. Hansen *et al.* (1972), Pawlowski & Vaughan (1972) and Vaughan & Hansen (1973), investigating the response of HeLa cells to histidine starvation and histidinol addition, found no evidence of premature termination in protein synthesis. Further, histidinol changed the polyribosome profile of the HeLa cells to a preponderance of ribosomes and small polyribosomes (Vaughan & Hansen, 1973), a finding that argues against uncharged tRNA slowing protein synthesis via a decrease in the peptide-elongation rate. Instead histidinol and the isoleucine-charging inhibitor, *O*-methylthreonine, lowered the rate of initiation in protein synthesis. However, Vaughan & Hansen (1973) mention data in a footnote whereby histidinol did increase the average size of polyribosomes in rabbit reticulocyte lysates, thus implicating a difference between HeLa cells and reticulocytes.

Rapid degradation of proteins synthesized in the presence of histidinol could occur either while the nascent peptide is still attached to the ribosome or after its release. Since the electrophoretic separation of peptides in Fig. 3 was carried out with total reticulocyte protein rather than with a ribosome-free lysate, no distinction can be made between the two possibilities. Unfortunately the time needed for lysis and centrifugation precluded the more definitive experiment, because substantial degradation could not be avoided. Certainly the proteolytic cleavage of abnormally shortened proteins or those with amino acid substitutions can occur after release of protein from ribosomes, because both puromycin peptides (McIlhinney & Hogan, 1974) and peptides with 2-thiazolylalanine replacing phenylalanine (S. E. Knowles & F. J. Ballard, unpublished work) are rapidly degraded in reticulocytes.

Since inhibition of protein synthesis in the absence of an essential amino acid or presence of histidinol gives rise to labile protein, we have investigated the effects of other protein-synthesis inhibitors. For this purpose we have chosen the elongation inhibitor, cycloheximide, and the initiation inhibitor, fluoride (Vasquez, 1974). At cycloheximide concentrations that decrease protein synthesis to the extent found in

Table 4. *Synthesis and subsequent degradation of proteins in the presence of inhibitors of protein synthesis*

Protein synthesis and degradation were measured in rabbit reticulocytes as described in the Materials and Methods section. Inhibitors were only present during the protein-labelling period. Degradation has been corrected for changes in the amount of ^3H -labelled leucyl-tRNA by treating the trichloroacetic acid-insoluble fraction with 0.5M-glycine, pH 10.

Labelling medium	Inhibitor	Protein synthesis (% of control)	Protein degradation (% in 2h)
Complete	—	100	3.3
	Cycloheximide (1 μM)	39	5.2
	Cycloheximide (3 μM)	21	5.7
	Cycloheximide (30 μM)	7	8.0
	NaF (10mM)	37	6.2
	NaF (30mM)	10	5.6
	Puromycin (50 μM)	8	59.5
Minus histidine	—	17	14.0
	Histidinol (3mM)	8	27.5

Table 5. *Effect of histidinol on the degradation of pre-existing protein in reticulocytes*

Rat reticulocytes were incubated in complete medium to label proteins as described in the Materials and Methods section, except that [^{14}C]leucine (0.2 $\mu\text{Ci/ml}$) replaced [^3H]leucine and non-radioactive leucine was not added. The cells were then washed, preincubated for 30min in either complete medium or histidine-free medium and then incubated for a further 30min in the appropriate medium with 10 μM -leucine and 6 μCi of [^3H]leucine per ml (second incubation). Histidinol (3mM) was added to the medium without histidine. Degradation of both ^{14}C -labelled protein and ^3H -labelled protein was subsequently measured over a 2h period. Values are the average of two experiments.

Second incubation conditions	Percentage degradation in 2h of labelled protein	
	^{14}C	^3H
Complete medium	1.32	4.39
Without histidine + 3mM-histidinol	1.16	35.3

medium lacking histidine a small amount of labile protein is formed (Table 4). However, even when protein synthesis was decreased to the same extent as

that found with 3 mM-histidinol, the effect on protein degradation was very small. Also NaF does not produce a large increase in labile protein when this inhibitor is added at concentrations that give equivalent inhibition of protein synthesis (Table 4). Consequently the response to amino acid imbalance appears relatively specific. In contrast with other protein-synthesis inhibitors, puromycin gives rise to much more labile protein (Table 4). Indeed 20% of the protein labelled in the presence of 50 μ M-puromycin is degraded during the first 2 min of reticulocyte incubation (Ballard, 1976), a result consistent with the report of McIlhinney & Hogan (1974). Thus the initial degradation rate of puromycyl peptides is much faster than that found for histidinol protein, a difference that might explain why the extensive degradation shown for puromycyl peptides in lysates (Etlinger & Goldberg, 1977) does not occur with protein labelled in the presence of histidinol.

In the present work we have not measured the extent to which tRNA is uncharged during amino acid starvation or when histidinol is added. Other workers have shown decreases in tRNA charging under less extreme conditions (Allen *et al.*, 1969) as well as with histidinol (Vaughan & Hansen, 1973). The histidinol concentrations we have used with reticulocytes (2 mM or 3 mM) inhibited protein synthesis to about the same extent as that found by

Vaughan & Hansen (1973) in HeLa cells, where the charging of tRNA was decreased by about 40% (Vaughan & Hansen, 1973). A comparable decrease in histidinyl-tRNA may thus be expected in reticulocytes.

Effect of histidinol on the degradation of existing protein

Amino acid starvation in bacteria leads to the breakdown of cell protein (Sussman & Gilvarg, 1969; Goldberg, 1971) as part of the pleiotypic response (Goldberg, 1971). We have attempted a similar approach in reticulocytes by first prelabelling cells with [14 C]leucine and then exposing them to [3 H]-leucine either in complete medium or in the presence of histidinol. Degradation of both 14 C-labelled protein and 3 H-labelled protein was subsequently followed. This experiment (Table 5) shows that incubation in deficient medium has no effect on the breakdown of pre-existing protein (14 C-labelled) in reticulocytes. We conclude that the degradation of the labile protein in reticulocytes is not related to the stringent response in bacteria.

Effect of proteolytic inhibitors on the degradation of protein synthesized in the presence of histidinol

Proteolytic inhibitors, as well as growth promoters such as foetal calf serum, inhibitors of protein synthesis and cell poisons all decrease the degradation rate of proteins in cultured cells (Knowles & Ballard, 1976). However, only fluoride and dinitrophenol decrease the degradation of reticulocyte protein (Table 6) and essentially equal effects are seen on protein made in complete medium and protein synthesized with histidinol present. Neither the cathepsin B inhibitor, leupeptin, nor the cathepsin D inhibitor, pepstatin, has a marked effect on protein breakdown. In this respect reticulocyte protein is similar to labile protein formed in cultured cells in the presence of the arginine analogue canavanine (Knowles & Ballard, 1976). Since it is probably lysosomal proteolysis that is inhibited by nutrients, protein-synthesis inhibitors and leupeptin (Knowles & Ballard, 1976), perhaps the breakdown of protein in reticulocytes does not take place in lysosomes. Whether this explanation holds, the mechanism responsible in reticulocytes seems similar for both normal and abnormal protein. Any further characterization of the degradation process requires a cell-free preparation in which protein formed in the presence of histidinol is degraded at appropriately rapid rates compared with normal protein. The ATP-requiring proteolytic system described by Etlinger & Goldberg (1977) may prove useful in this regard.

Table 6. *Effects of inhibitors on the breakdown of normal protein and protein synthesized in the presence of histidinol*

Rabbit reticulocytes were labelled with [3 H]leucine in either complete medium or minus histidine medium supplemented with 3 mM-histidinol as described in the Materials and Methods section. The cells were washed and incubated for 2 h in complete medium with the inhibitors added at the concentrations indicated.

Inhibitor	Percentage degradation in 2 h of protein labelled in	
	Complete medium	Medium minus histidine plus histidinol
None	3.7	34.1
Cycloheximide (30 μ M)	4.0	36.0
NaF (30 mM)	1.9	18.9
Foetal calf serum (10%)	4.0	33.8
Dinitrophenol (0.1 mM)	0.9	12.6
Leupeptin (50 μ g/ml)	3.2	34.4
Pepstatin (50 μ g/ml)	3.7	31.5
Phenylmethanesulphonyl fluoride (2.5 mM)	3.4	25.9

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