Mechanisms of intracellular protein catabolism

Intracellular fate of microinjected polypeptides translated *in vitro*

Michael J. GASKELL,* Peter C. HEINRICH† and R. John MAYER*

*Department of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH, U.K., and †Biochemisches Institut, Albert Ludwigs Universität, Herman-Herder Strasse 7, D7800 Freiburg-i-Br., West Germany

Erythrocyte-mediated microinjection was used to introduce [³⁵S]polypeptides translated *in vitro* into 3T3-L1 cells. Such [³⁵S]polypeptides are not degraded after loading into erythrocytes and are stable for the first 2 h after microinjection into growing 3T3-L1 cells. Similarly, little or no degradation of microinjected [³⁵S]polypeptides is observed in either growing or confluent 3T3-L1 cells over a 70 h period. Microinjection of reticulocyte lysate alone does not affect the rate of degradation of long-lived endogenous protein. Reductively [³H]methylated lysate haemoglobin is degraded after microinjection by a cytosolic mechanism. Microinjected ¹²⁵I-labelled bovine serum albumin is rapidly degraded by a cytosolic mechanism at the same rate in the absence or presence of reticulocyte lysate. The data do not support the notion that the observed lack of degradation of microinjected [³⁵S]polypeptides translated *in vitro* is due to the presence of proteolytic inhibitors in reticulocyte lysates which can inhibit the degradation of microinjected or cellular proteins.

INTRODUCTION

The mechanisms of intracellular protein degradation, including the molecular criteria by which a protein is targeted to a degradative system, are not understood. Protein degradation has been demonstrated to occur both lysosomally and non-lysosomally (Dean, 1975; Amenta & Brocher, 1981; Hendil, 1981; Rote & Rechsteiner, 1983; McElligott & Dice, 1983; Doherty & Mayer, 1985a,b). In contrast with protein synthesis, it is very difficult at present to define the intracellular site of degradation of a single protein or group of proteins.

In the last few years techniques such as erythrocyte-mediated microinjection (Schlegel & Rechsteiner, 1975), needle microinjection (Graessmann & Graessmann, 1971) and scrape-loading (McNeil *et al.*, 1984) have been developed which enable specific radiolabelled proteins to be introduced into unlabelled target tissue-culture cells so that their degradation may be studied. Several reports describe the degradation of proteins microinjected into cells (Hendil, 1980; Dice, 1982; Hough & Rechsteiner, 1984; Katznelson & Kulka, 1985; Doherty & Mayer, 1985a), but mature processed proteins have been routinely used in these studies. We have therefore studied the intracellular fate of microinjected [³⁵S]polypeptides translated *in vitro* (consisting of precursor polypeptides lacking co- and post-translational modifications). Differences in the intracellular processing of microinjected precursor polypeptides, compared with microinjected mature proteins, are valuable in order to define the molecular criteria for targeting proteins into the intracellular degradative systems.

MATERIALS AND METHODS

Materials

3T3-L1 pre-adipocytes were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium was supplied by Gibco Bio-Cult (Paisley, Scotland, U.K.)

and 90 mm-diam. plastic tissue-culture dishes were from Nunc (Kamstrup, Denmark). Newborn-calf serum was supplied by Imperial Laboratories (Salisbury, Wilts., U.K.). All radioisotopes were from Amersham International (Amersham, Bucks., U.K.), and penicillin G, streptomycin, trypsin, oligo(dT)-cellulose and protein *M_r* standards were from Sigma Chemical Co. (Poole, Dorset, U.K.). Fisofluor was obtained from Fisons (Loughborough, Leics., U.K.) and pre-packed Sephadex G-25 columns were from Pharmacia Fine Chemicals (Hounslow, Middx., U.K.).

Tissue culture

3T3-L1 cells were cultured as described by Doherty & Mayer (1985a).

Radiolabelling of proteins

3T3-L1 monolayers were radiolabelled metabolically by culture with [³⁵S]methionine (1 µCi/ml of culture medium). Cells were then washed, removed for sub-cultivation by incubation with trypsin and resuspended in Dulbecco's modified Eagle's medium plus 10% (v/v) newborn-calf serum, antibiotics and 10 mM-methionine. Haemoglobin in reticulocyte lysates was reductively methylated with NaB³H₄ by the method of Tack *et al.* (1980). Bovine serum albumin was radioiodinated with Na¹²⁵I by the method of Hunter & Greenwood (1962).

mRNA isolation

Liver mRNA was prepared by the method of Aviv & Leder (1972).

Translation of radiolabelled polypeptides *in vitro*

The mRNA-dependent rabbit reticulocyte lysate was prepared by the method of Pelham & Jackson (1976). Radiolabelled polypeptides were synthesized in the mRNA-dependent reticulocyte lysate in the presence of [³⁵S]methionine by the method of Pelham & Jackson (1976) as modified by Andus *et al.* (1983).

Preparation of protein samples for microinjection

Reticulocyte lysates (usually ten 50 μ l translation mixtures) containing [35 S]polypeptides were pooled and layered on to 1 ml of 1.2 M-sucrose (adjusted to pH 7.4 with NaHCO_3) in a 2 ml insert tube of a 10 $\times$ 10 ml MSE centrifuge rotor. Ribosomes in the reticulocyte lysate were sedimented by centrifugation at 180 000 g_{av} for 45 min. The red supernatant on the sucrose cushion was removed and applied to a Sephadex G-25 column (1 cm $\times$ 10 cm) pre-equilibrated with 10 mM- NaH_2PO_4 buffer, pH 7.4, containing 10 mM-methionine. The red void-volume fraction was collected. This procedure removes > 98% of the free [35 S]methionine present in the reticulocyte lysate. The lysate was concentrated to the desired volume for loading into erythrocytes by ultrafiltration in an Amicon (Stonehouse, Gloucs., U.K.) stirred cell with a 10 000-Da filter.

Microinjection

Radiolabelled polypeptides were loaded into human erythrocytes and microinjected into 3T3-L1 cells by using Sendai virus by the method of Schlegel & Rechsteiner (1975) as modified by Doherty & Mayer (1985a). After fusion at 37 $^{\circ}\text{C}$, cells were pelleted at 500 g for 2 min, suspended in 2 ml of 0.83% (w/v) NH_4Cl (at 4 $^{\circ}\text{C}$), to lyse red blood cells, layered on to 8 ml of a 1:1 (v/v) mixture of newborn-calf serum and 0.83% NH_4Cl in a pointed sterile tube, and centrifuged at 500 g for 2 min. The washing procedure was repeated once, and the cells were then resuspended in growth medium at 37 $^{\circ}\text{C}$ for immediate use.

Measurement of protein degradation

Protein degradation was measured either by the method described by Doherty & Mayer (1985a) or from the rate of loss of cell-associated trichloroacetic acid (final concn. 10%, w/v)-insoluble radioactivity.

Enzyme activities

The enzyme assays carried out on each detergent/salt fraction were lactate dehydrogenase (EC 1.1.1.27) (Johnson, 1960), acid phosphatase (EC 3.1.3.2) (de Duve *et al.*, 1955) and succinate dehydrogenase (EC 1.3.99.1) (Pennington, 1961). Relative protein concentration (i.e. percentage of radiolabelled protein) in the detergent/salt fractions were estimated after prolonged metabolic radiolabelling with [35 S]methionine (48 h). DNA content was estimated after prolonged metabolic radiolabelling with [^3H]thymidine (24 h).

RESULTS

Stimulation of protein synthesis in rabbit reticulocyte lysates by exogenous rat liver mRNA

Fig. 1 shows densitometric scans of fluorograms of polyacrylamide gels after electrophoresis of ^{35}S -radiolabelled polypeptides from reticulocyte lysates. Such [^{35}S]polypeptides translated *in vitro* from mRNA-dependent lysates (Fig. 1a) were routinely loaded into erythrocytes and microinjected into 3T3-L1 cells.

[^{35}S]Polypeptides translated *in vitro* are predominantly stable in erythrocytes

Fig. 2 demonstrates that ^{35}S -labelled precursor polypeptides translated *in vitro* are only degraded to a

Fig. 1. Densitometry scans of fluorograms of ^{35}S -radiolabelled polypeptides in reticulocyte lysates

(a) With mRNA (3 μg); (b) without mRNA. The translations *in vitro* were carried out in the presence of approx. 50 μCi of [^{35}S]methionine. Positions of M_r markers ($\times 10^{-3}$) are indicated at the top of each panel. Fluorograms were scanned with white light with a Helena Quick Scan R+D Scanner (Helena Laboratories, Beaumont, TX, U.S.A.).

small extent (approx. 22%) after loading into erythrocytes before microinjection. In all subsequent experiments, loaded erythrocytes were fused to 3T3-L1 target cells within 1 h of loading.

Residual [^{35}S]methionine is not incorporated into 3T3-L1 cell protein during fusion with loaded erythrocytes in the presence of 10 mM-methionine

Although chromatography on Sephadex G-25 removes 98% or more of free [^{35}S]methionine from reticulocyte lysates, loaded erythrocytes may still contain [^{35}S]methionine (up to 1.5 $\mu\text{Ci}/\text{ml}$). Unless excess of competing unlabelled methionine is present in the fusion buffer, [^{35}S]methionine will become incorporated into 3T3-L1 cell protein when the loaded erythrocytes are fused with the 3T3-L1 target cells. The effect of

Fig. 2. Stability in erythrocytes of [³⁵S]polypeptides translated *in vitro*

Samples (5 μ l) were taken at intervals from 100 μ l of erythrocytes in 100 μ l of Hanks Balanced Salt Solution. Erythrocytes were loaded with 150 000 d.p.m. of trichloroacetic acid-insoluble labelled material and incubated at 37 °C. Results are expressed as means $\pm$ half of the difference between duplicate measurements.

competing 10 mM-methionine on incorporation of [³⁵S]methionine into 3T3-L1 cell protein is shown in Fig. 3. 3T3-L1 cells were incubated with [³⁵S]methionine in fusion conditions, i.e. 0 °C for 10 min, then at 37 °C for 20 min. In the presence of 10 mM-methionine there is very little, if any, incorporation of [³⁵S]methionine into trichloroacetic acid-insoluble material. In the absence of competing 10 mM-methionine, there is a large incorporation of [³⁵S]methionine into 3T3-L1 cell protein during the 20 min incubation at 37 °C. This decreases during the following 60 min as a portion of the newly synthesized protein is degraded (Bienkowski, 1983; see Fig. 4). Incorporation of [³⁵S]methionine into 3T3-L1 cell protein in the absence of 10 mM-methionine would obscure measurements on the fate of microinjected [³⁵S]polypeptides translated *in vitro* (see below).

Microinjected [³⁵S]polypeptides translated *in vitro* are stable in 3T3-L1 cells for at least 2 h after microinjection

Microinjected polypeptides translated *in vitro* may be degraded very rapidly in 3T3-L1 cells, e.g. by mechanisms which degrade amino-acid-analogue-containing proteins (Goldberg & Dice, 1974; Knowles & Ballard, 1976; Wharton & Hipkiss, 1984; Ciechanover *et al.*, 1984). Fig 4 shows the cell-associated trichloroacetic acid-insoluble radioactivity in 3T3-L1 cells in suspension at 37 °C over a 2 h period. In this experiment microinjected cells were resuspended after washing at 4 °C (see the Materials and methods section) in warm (37 °C) medium and the cell suspension was used immediately. Endogenous ³⁵S-radiolabelled proteins ($\blacktriangle$) and endogenous proteins radiolabelled (cf. Fig. 3) from residual [³⁵S]methionine in reticulocyte lysate in the absence of added 10 mM-

Fig. 3. Incorporation of [³⁵S]methionine into 3T3-L1 cell protein

3T3-L1 cells at a density of 10⁷ cells/ml in suspension were incubated in the presence (○) or absence (●) of 10 mM-methionine with [³⁵S]methionine (1.5 μ Ci/ml). Cells were incubated for 10 min at 0 °C and 20 min at 37 °C in the presence of [³⁵S]methionine. Cells were washed in Dulbecco's medium, centrifuged and resuspended in Dulbecco's medium for 60 min at 37 °C. Values are expressed as means $\pm$ half of the difference between two measurements.

methionine (●) undergo rapid degradation in 3T3-L1 cells. In the presence of 10 mM-methionine (○) there is no measurable loss of trichloroacetic acid-insoluble radiolabel from cells microinjected with [³⁵S]polypeptide translated *in vitro* over short periods. The microinjected [³⁵S]polypeptides are not subject to very rapid protein catabolism.

Every precaution was subsequently taken to avoid the incorporation of residual lysate [³⁵S]methionine into endogenous cell protein. All solutions, buffers and media used in experiments with [³⁵S]polypeptides translated *in vitro* were made 10 mM in methionine.

Degradation of microinjected [³⁵S]polypeptides cannot be measured in growing or confluent 3T3-L1 cell monolayers

The long-term fate of microinjected [³⁵S]polypeptides was studied in growing and confluent 3T3-L1 cells by fusing loaded erythrocytes with 3T3-L1 cells, then seeding the microinjected cells into 90 mm culture dishes at either growing (10⁴ cells/cm²) or confluent (5 $\times$ 10⁴ cells/cm²) cell densities. If insufficient microinjected radiolabelled cells were present to cause confluence, an appropriate number of non-injected cells was added to each plate to ensure confluence. There is no degradation (Fig. 5) of the [³⁵S]polypeptides translated *in vitro* in growing or confluent 3T3-L1 cells.

Fig. 4. Cell-associated trichloroacetic acid-insoluble radioactivity in 3T3-L1 cells in suspension after microinjection of [^{35}S]polypeptides translated *in vitro* or radiolabelling of endogenous cellular proteins

For endogenous radiolabelling, [^{35}S]methionine equivalent to the maximum amount of the radioisomer ever observed in reticulocyte lysate after Sephadex G-25 filtration (1.5 $\mu\text{Ci}/\text{ml}$) was incubated with 3T3-L1 cells in exactly the same conditions as for the fusion procedure. ○, Microinjected 3T3-L1 cells with 10 mM-methionine in Dulbecco's medium. ●, Microinjected 3T3-L1 cells without 10 mM-methionine in Dulbecco's medium. ▲, Endogenously labelled 3T3-L1 cells. Samples (100 μl) from microinjected or endogenously radiolabelled cells (2 ml; 10^7 cells/ml) were taken for analysis. Results are expressed as the percentage of total trichloroacetic acid-insoluble radioactivity at zero time. The mean radioactivity at zero time was (○) 4505 d.p.m., (●) 1266 d.p.m., (▲) 5825 d.p.m. The results represent means $\pm$ half the difference between duplicate measurements in separate experiments, i.e. performed on different occasions with different lysates. Microinjection efficiency, i.e. loading of radioactivity into target cells, varied from 34% (○) to 10% (●) in different experiments [routinely efficiencies of 6–11% (Fig. 5) were obtained].

Subcellular distribution of microinjected [^{35}S]polypeptides

The observation that microinjected [^{35}S]polypeptides are apparently not degraded over 70 h in both growing and confluent cells is very unusual, previously only seen in cases such as the very slow degradation of extracellular proteins, e.g. collagen (Jackson, 1973). The subcellular distribution of microinjected [^{35}S]polypeptides was evaluated by sequential detergent/salt-extraction procedures (Doherty *et al.*, 1987; Earl *et al.*, 1987b). In growing cells similar amounts of

Fig. 5. Stability of microinjected [^{35}S]polypeptides in growing (a) and confluent (b) monolayer cultures of 3T3-L1 cells

●, Trichloroacetic acid-insoluble radiolabel in cells; ○, trichloroacetic acid-soluble radiolabel in Dulbecco's medium. The data represent typical results from an experiment repeated four times. Results are expressed as means $\pm$ s.e.m. for triplicate measurements (a) or means $\pm$ half the difference between duplicate observations (b). Efficiency of microinjection, i.e. loading of radioactivity into target cells, was 6–11%. Measurements were commenced either immediately after fusion (b) or when the cells had attached to the plates (a).

[^{35}S]polypeptides were found in fractions extracted with digitonin, Triton X-100 and KI as well as in the non-extractable residue: in confluent cells the [^{35}S]polypeptides were mainly extractable (60–80%) by digitonin, implying a cytosolic distribution, similar to the marker lactate dehydrogenase or cytosolic protein (Fig. 9; Doherty & Mayer, 1985a). However, irrespective of subcellular distribution, no degradation of [^{35}S]polypeptides translated *in vitro* could be measured.

The observed lack of degradation of such polypeptides could be due to the presence of inhibitors of protein catabolism in reticulocyte lysates. The experiments described below discount this possibility.

Microinjection of reticulocyte lysate does not affect endogenous protein degradation

Fig. 6 shows the effect on endogenous protein degradation of microinjected reticulocyte lysate. Proteins in metabolically radiolabelled cells (○) are degraded with

Fig. 6. Degradation of endogenous proteins in 3T3-L1 cells after microinjection with reticulocyte lysate

3T3-L1 cells were prelabelled for 48 h with [^{35}S]methionine (1 $\mu\text{Ci}/\text{ml}$ of culture medium), microinjected with reticulocyte lysate and seeded out at 10^4 cells/ cm^2 . Non-microinjected cells were seeded out at 10^4 cells/ cm^2 . ●, Microinjected cells; ○, non-microinjected cells. Half-lives ($t_{1/2}$) were calculated by the method of Doherty & Mayer (1985a). The cell-associated trichloroacetic acid-insoluble radioactivity at zero time was calculated, and the trichloroacetic-soluble material in the medium at each time point was expressed as a percentage of this value. The rate constant of degradation (K_D) was calculated from the slope of the line $\ln(100\text{-percentage in medium})$ versus time: $t_{1/2} = \ln 2/K_D$. Original data points were expressed as means $\pm$ S.E.M. of quadruplicate observations.

an average half-life of 63 h. Proteins in metabolically radiolabelled cells microinjected with reticulocyte lysate (●) are degraded with a half-life of 59 h. The half-lives are not significantly different. The average half-life of proteins in 3T3-L1 cells was previously determined as 66 h (Doherty & Mayer, 1985a).

Microinjected [^3H]haemoglobin in reticulocyte lysate is degraded with biphasic kinetics

Reticulocyte lysate containing [^3H]haemoglobin was loaded into erythrocytes and microinjected into 3T3-L1 cells. Microinjected 3T3-L1 cells were seeded out at a density of 10^4 cells/ cm^2 . Reductive methylation (Tack *et al.*, 1980) of reticulocyte lysate with NaB^3H_4 modified haemoglobin almost exclusively (> 95% of radiolabel, as

shown by polyacrylamide-gel electrophoresis under denaturing conditions; results not shown). Fig. 7 shows that the loss of cell-associated trichloroacetic acid-insoluble radiolabel shows complex kinetics. Transformation of the data to $\ln$ radioactivity (d.p.m.) against time (Fig. 7, insert) shows that, initially, microinjected [^3H]haemoglobin is degraded with a half-life of approx. 173 h. As cell density increases, the half-life decreases to 65 h. The increase in rate occurs after approx. 45 h, at which time the 3T3-L1 cells are over 50% confluent (results not shown). A more complex interpretation of Fig. 7 (insert) might indicate three-phase degradation kinetics (degradation is initially slow, then faster in rapidly growing cells, then again slower in confluent cells).

Microinjected [^3H]haemoglobin is located in the digitonin-extractable cytosol throughout degradation

Sequential detergent/salt extraction (Doherty *et al.*, 1987) of microinjected 3T3-L1 cells at each time point shows that microinjected [^3H]haemoglobin is predominantly found (> 80%; Fig. 8) in the digitonin-extractable cytosol (Fig. 9). NH_4Cl (10 mM) has no effect on the degradation of microinjected [^3H]haemoglobin or on its subcellular location in the cytosol (results not shown).

Microinjected ^{125}I -labelled bovine serum albumin is degraded at similar rates in the presence and absence of co-microinjected reticulocyte lysate

Bovine serum albumin ^{125}I -labelled was microinjected into 3T3-L1 cells in the presence and absence of reticulocyte lysate to observe any differences in albumin degradation. Microinjected cells were seeded into culture dishes at 10^4 cells/ cm^2 . Fig. 10 shows the degradation of microinjected ^{125}I -labelled bovine serum albumin in the presence (●) and absence (○) of co-microinjected reticulocyte lysate. The calculated half-lives of 18½ and 18 h respectively are in good agreement with the half-life of 21 h reported by Doherty & Mayer (1985b). The degradation of microinjected ^{125}I -labelled bovine serum albumin is insensitive to 10 mM- NH_4Cl (Rote & Rechsteiner, 1983; Doherty & Mayer, 1985b). The microinjected ^{125}I -labelled bovine serum albumin (like haemoglobin) is predominantly (> 80%) in the digitonin-extractable cytosol (Fig. 9) throughout degradation. A more complex interpretation of Fig. 10 (insert) might indicate two-phase degradation kinetics (initial fast degradation, with slower subsequent degradation).

DISCUSSION

Netland & Dice (1985) suggested four criteria that should be considered before using erythrocyte-mediated microinjection to introduce radiolabelled proteins into target cells. These are: (i) there should be minimal alteration of the structure and function of a protein by the radiolabelling procedure; (ii) there should be little or no binding of protein to the outside of the erythrocyte membrane when loading; (iii) there should be no significant binding of radiolabelled proteins from lysed erythrocytes to the target cells; (iv) loaded protein should not be degraded after loading into the erythrocyte.

We have shown that [^{35}S]polypeptides translated *in vitro* are not significantly degraded after loading into

Fig. 7. Degradation of [^3H]haemoglobin in microinjected reticulocyte lysate in 3T3-L1 cells

Cell-associated trichloroacetic acid-insoluble radioactivity was measured. Each value represents the mean $\pm$ S.E.M. for triplicate observations. The insert shows transformation to $\ln[\text{radioactivity (d.p.m.)}]$ against time.

Fig. 8. Detergent/salt extraction of microinjected [^3H]haemoglobin from 3T3-L1 cells

Cells were extracted at intervals for 121 h after microinjection: (a) 0 h; (b) 4 h; (c) 22 h; (d) 46 h; (e) 70 h; (f) 97 h; (g) 121 h. Fractions: D, digitonin-extractable; T, Triton X-100-extractable; KI, KI-extractable; R, non-extractable residue. Results are expressed as the trichloroacetic acid-insoluble radioactivity present in each fraction. No errors are shown where they are too small to insert.

erythrocytes, before fusion with 3T3-L1 cells (Fig. 2). Such polypeptides are radiolabelled by incorporation of [^{35}S]methionine, so they are minimal deviants (^{35}S for ^{32}S) of endogenous cellular precursor polypeptides. Incorporation of [^{35}S]methionine should therefore have the smallest effects on protein structure. We have also shown that [^{35}S]polypeptides translated *in vitro* loaded into erythrocytes are insensitive to external trypsin, and that the proportion of cell-associated trichloroacetic acid-insoluble radioactivity in microinjected 3T3-L1 cells

owing to binding of the contents of lysed loaded erythrocytes does not exceed 20% (results not shown). These results satisfy the criteria of Netland & Dice (1985).

The observation that there is little degradation of loaded [^{35}S]polypeptides in erythrocytes (Fig. 2) is very important because of the demonstration of several degradative systems in reticulocyte lysates and erythrocytes. Hershko *et al.* (1978) have characterized an ATP- and ubiquitin-dependent proteolytic system in reticulo-

Fig. 9. Enzyme, protein and DNA distributions in detergent/salt extracts from 3T3-L1 cells

Fractions: D, Digitonin extract; T, Triton X-100 extract; KI, KI extract; R, non-extractable residue. Enzyme activities are expressed as the percentage of the total activity observed in the sum of the three extracts and residue. Protein and DNA distributions are estimated as the trichloroacetic acid-insoluble material in each extract and residue after prolonged cell labelling with [35 S]methionine (1 μ Ci/ml of culture medium for 48 h) and [3 H]thymidine (1 μ Ci/ml of culture medium for 24 h) respectively. Results are expressed as means $\pm$ half the difference between the duplicate measurements. No errors are shown where they were too small to insert.

cyte lysates, and Speiser & Etlinger (1982) have shown that it is possible to activate a very similar system in erythrocytes. An ATP-independent system has also been demonstrated in reticulocyte lysate (Speiser & Etlinger, 1983), and Pontremoli *et al.* (1984) have reported a Ca^{2+} -dependent proteinase associated with the degradation of erythrocyte membrane proteins. In spite of the possibility of action of one or more of these proteolytic mechanisms, [35 S]polypeptides translated *in vitro* are apparently not significantly degraded over 1 h after loading into erythrocytes.

An equally important observation is that [35 S]polypeptides translated *in vitro* are stable over short periods after microinjection into 3T3-L1 cells (Fig. 4), despite the existence of characterized systems for the degradation of amino-acid-analogue-containing abnormal proteins (Goldberg & Dice, 1974; Knowles & Ballard, 1976; Wharton & Hipkiss, 1984) which may be associated with a ubiquitin-dependent pathway (Ciechanover *et al.*, 1984). We cannot exclude the possibility that some degradation of [35 S]proteins translated *in vitro* occurs before the commencement of measurements, i.e. during fusion, leaving residual 'undegradable' polypeptides, although the washing of cells after fusion in solutions at 4 $^{\circ}\text{C}$ would preclude temperature-dependent degradation processes. The results indicate that [35 S]polypeptides translated *in vitro* are

refractory to reported reticulocyte, erythrocyte and cellular degradation systems for amino-acid-analogue-containing proteins. Furthermore, little or no degradation of microinjected precursor [35 S]polypeptides translated *in vitro* (Fig. 6) occurs in both growing and confluent 3T3-L1 cells over a 70 h time period. This is in marked contrast with all other microinjected mature proteins; see, e.g., Zavortinck *et al.* (1979), Hendil (1980), Rote & Rechsteiner (1983) and Doherty & Mayer (1985a).

Microinjection of reticulocyte lysate into growing 3T3-L1 cells caused little alteration to the rate of endogenous-protein degradation (Fig. 6). It has previously been demonstrated that erythrocyte microinjected homologous cytosol mixtures (Doherty & Mayer, 1985a) and single proteins (Hendil, 1980; Backer *et al.*, 1983; McElligott & Dice, 1984) do not alter the rate of endogenous protein degradation.

It is possible that the lysate contains inhibitors of some specific proteolytic mechanism required for degradation of proteins in reticulocyte lysate. This possibility was investigated for lysate haemoglobin after reductive methylation with NaB^3H_4 . The results (Fig. 7) show multi-phasic kinetics for the degradation of [3 H]haemoglobin after microinjection in reticulocyte lysate. The simplest interpretation is that haemoglobin is degraded slowly ($t_{1/2}$ 173 h) after microinjection until approx. 45 h, whereafter the protein is degraded more

Fig. 10. Degradation of microinjected ^{125}I -labelled bovine serum albumin in 3T3-L1 cells

^{125}I -labelled bovine serum albumin was microinjected into 3T3-L1 cells in the presence (●) and absence (○) of reticulocyte lysate. Cells were sampled over 70 h. Each value represents the mean $\pm$ half the difference between duplicate observations. The inset shows transformation of data to $\ln[\text{radioactivity (d.p.m.)}]$ against time.

than twice as fast ($t_{1/2}$ 65 h). The relatively slow initial rate of degradation could reflect the presence of inhibitors, from either reticulocyte lysate or the erythrocyte, acting initially only on microinjected lysate proteins, which are subsequently gradually diluted out, e.g. by cell growth or inhibitor degradation. Alternatively, microinjected ^3H haemoglobin may be slowly degraded in rapidly growing cells and more quickly degraded in cells approaching confluency. For example, autophagy is known to be induced as cell density increases (Hendil, 1981; Amenta & Brocher, 1981; Mayer & Doherty, 1986). The erythrocyte is an unlikely source of inhibitors, since many proteins have been microinjected into cells by using erythrocytes and degraded with simple kinetics, e.g. bovine serum albumin (Fig. 10; Rote & Rechsteiner, 1983; Doherty & Mayer, 1985b), RNAase (Backer *et al.*, 1983; McElligott *et al.*, 1985) and several other proteins (Rogers & Rechsteiner, 1985).

Fig. 10 shows no significant difference in half-life ($t_{1/2}$ approx. 18 h) for ^{125}I -labelled bovine serum albumin microinjected into 3T3-L1 cells in the presence and absence of reticulocyte lysate, which suggests that the lysate does not contain proteolytic inhibitors. Hendil (1980) labelled haemoglobin in erythrocytes *in situ* by ^{14}C carbamoylation and then microinjected the radio-labelled haemoglobin into BHK-21 fibroblasts. In sparse,

actively growing, cells microinjected ^{14}C haemoglobin is degraded slowly ($t_{1/2}$ 164 h). At high culture density or during serum deprivation, the degradative rate doubled ($t_{1/2}$ 84 h). Erythrocyte haemoglobin (not reticulocyte-lysate haemoglobin) was injected in those studies, with very similar degradation characteristics observed to those reported here (Fig. 7), i.e. biphasic degradation. We therefore conclude, as did Hendil (1980), for erythrocyte haemoglobin, that the increase in degradative rate of microinjected lysate ^3H haemoglobin is due to the target cells approaching confluency.

More than 80% of microinjected ^3H haemoglobin is in the digitonin-extractable cytosol of 3T3-L1 cells throughout degradation (cf. Figs. 8 and 9). The protein is also predominantly found in the cytosolic fraction prepared by differential centrifugation of homogenates of IMR-90 fibroblasts after microinjection with ^{14}C haemoglobin (Slot *et al.*, 1986). These complementary data support the digitonin-extractability of cytosol from cells (Mackall *et al.*, 1979; Earl *et al.*, 1987b). Microinjected ^3H haemoglobin remains in the cytosol throughout degradation (Fig. 8), implying catabolism by a cytosolic degradation system. This notion is supported by preliminary experiments showing the lack of effect of NH_4Cl on ^3H haemoglobin degradation (results not shown). Cytosolic degradation of injected haemoglobin is also supported by the results of Stacey & Allfrey (1977) and Hendil (1980), who were unable to demonstrate any association of microinjected haemoglobin with lysosomes. The complex degradation kinetics for haemoglobin are therefore not due to enhanced autophagic degradation as cells approach confluency.

Degradation of microinjected ^{125}I -labelled bovine serum albumin is also insensitive to NH_4Cl (Rote & Rechsteiner, 1983; Doherty & Mayer, 1985b). Over 80% of microinjected ^{125}I -labelled bovine serum albumin is in the digitonin-extractable cytosol (results not shown), and albumin remains in the cytosol throughout degradation. This implies catabolism by a cytosolic degradative system, though not necessarily the same cytosolic system that degrades microinjected ^3H haemoglobin (Fig. 7), since ^{125}I -labelled bovine serum albumin is degraded with different kinetics (Fig. 10). Complex degradation kinetics for injected proteins similar to those described here have been noted previously (Rechsteiner *et al.*, 1984).

Overall, the experiments reported here provide no data to support the idea that reticulocyte lysate contains proteolytic inhibitors capable of preventing the degradation of microinjected ^{35}S polypeptides translated *in vitro*.

^{35}S Polypeptides synthesized *in vitro* translated from liver mRNA are essentially unprocessed, e.g. retaining leader sequences and lacking prosthetic groups (Clemens, 1984). Microinjected polypeptides in such a protein mixture translated *in vitro* may therefore be unable to gain access to functional sites, and retain atypical conformations in target cells. Secretory-protein mRNA constitutes approx. 28% of total liver mRNA (Scornik & Botbol, 1976). Secretory proteins translated *in vitro* will contain cleavable leader sequences and may be mis-compartmented in the cell after microinjection. Therefore most microinjected ^{35}S polypeptides translated *in vitro* may have structures and conformations which render them refractory to normal intracellular degradative processes.

Note added in proof (received 14 November 1986)

Bachmair *et al.* (1986) have provided evidence that the nature of the *N*-terminal amino acids of β -galactosidases expressed from transfected mutant genes in yeast determines enzyme degradation rate. The '*N*-end rule' of Bachmair *et al.* (1986) is supported and extended by the observations reported here, which suggest that *N*-terminal sequences may control protein degradation even to the extent of rendering proteins refractory to degradative mechanisms: the question remains as to how this is achieved.

We thank the S.E.R.C. and N.A.T.O. for financial support, Mrs. Julie Wassell for drawing the Figures and Mrs. Millie Spooner for typing the manuscript.

REFERENCES

- Amenta, J. S. & Brocher, S. C. (1981) *Life Sci.* **28**, 1195–1208
- Andus, T., Gross, V., Tran-Thi, T.-A. & Heinrich, P. C. (1983) *FEBS Lett.* **151**, 10–14
- Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408–1412
- Backer, J. M., Bourrett, L. & Dice, J. F. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2166–2170
- Bachmair, A., Finley, D. & Varshavsky, A. (1986) *Science* **234**, 179–186
- Bienkowski, R. S. (1983) *Biochem. J.* **214**, 1–10
- Ciechanover, A., Finley, D. & Varshavsky, A. (1984) *Cell* **37**, 57–66
- Clemens, M. J. (1984) in *Transcription and Translation, a Practical Approach* (Hames, B. D. & Higgins, S. J., eds.), pp. 231–270, IRL, Oxford
- Dean, R. T. (1975) *Nature (London)* **257**, 414–416
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Dice, J. F. (1982) *J. Biol. Chem.* **257**, 14624–14627
- Doherty, F. J. & Mayer, R. J. (1985a) *Biochem. J.* **226**, 685–695
- Doherty, F. J. & Mayer, R. J. (1985b) *Biochem Soc. Trans.* **13**, 1170–1172
- Doherty, F. J., Wassell, J. A. & Mayer, R. J. (1987) *Biochem. J.* **241**, 793–800
- Earl, R. T., Billett, E. E., Hunneyball, I. M. & Mayer, R. J. (1987a) *Biochem. J.* **241**, 801–807
- Earl, R. T., Mangiapane, E. H., Billett, E. E. & Mayer, R. J. (1987b) *Biochem. J.* **241**, 809–815
- Goldberg, A. L. & Dice, J. F. (1974) *Annu. Rev. Biochem.* **43**, 835–869
- Graessmann, A. & Graessmann, M. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 527–532
- Hendil, K. B. (1980) *J. Cell. Physiol.* **105**, 449–460
- Hendil, K. B. (1981) *Exp. Cell Res.* **135**, 157–166
- Hershko, A., Heller, H., Ganoth, D. & Ciechanover, A. (1978) in *Protein Turnover and Lysosome Function* (Segal, H. L. & Doyle, D. J., eds.), pp. 149–169, Academic Press, New York
- Hough, R. & Rechsteiner, M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 90–94
- Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496
- Jackson, D. S. (1973) in *Biology of Fibroblast* (Kulonen, E. & Pikkariainen, J., eds.), pp. 417–420, Academic Press, London
- Johnson, M. K. (1960) *Biochem. J.* **77**, 610–618
- Katznelson, R. & Kulka, R. G. (1985) *Eur. J. Biochem.* **146**, 437–442
- Knowles, S. E. & Ballard, F. J. (1976) *Biochem. J.* **156**, 609–617
- Lazarides, E. (1980) *Nature (London)* **283**, 249–256
- Mackall, J., Meredith, M. & Lane, M. D. (1979) *Anal. Biochem.* **95**, 270–274
- Mayer, R. J. & Doherty, F. (1986) *FEBS Lett.* **198**, 181–193
- McElligott, M. A. & Dice, J. F. (1983) *Biochem. J.* **216**, 559–566
- McElligott, M. A. & Dice, J. F. (1984) *Biosci. Rep.* **4**, 451–466
- McElligott, M. A., Miao, P. & Dice, J. F. (1985) *J. Biol. Chem.* **260**, 11986–11993
- McNeil, P. L., Murphy, R. F., Launi, F. & Taylor, D. L. (1984) *J. Cell Biol.* **98**, 1556–1564
- Netland, P. A. & Dice, J. F. (1985) *Anal. Biochem.* **150**, 214–220
- Pelham, H. R. B. & Jackson, E. J. (1976) *Eur. J. Biochem.* **67**, 247–256
- Pennington, R. J. (1961) *Biochem. J.* **80**, 649–654
- Pontremoli, S., Melloni, E., Sparatore, B., Michetti, M. & Horecker, B. L. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6714–6717
- Rechsteiner, M., Chin, D., Hough, R., McGarry, T., Rogers, S., Rote, K. & Wu, L. (1984) *Cell Fusion: Ciba Found. Symp.* **103**, 181–207
- Rogers, S. W. & Rechsteiner, M. C. (1985) *Intracellular Protein Catabolism: Prog. Clin. Biol. Res.* **180**, 405–416
- Rote, K. V. & Rechsteiner, M. (1983) *J. Cell. Physiol.* **116**, 103–110
- Schlegel, R. & Rechsteiner, M. (1975) *Cell* **5**, 371–379
- Scornik, O. A. & Botbol, V. (1976) *J. Biol. Chem.* **251**, 2891–2897
- Slot, L. A., Lauridsen, A.-M. B. & Hendil, K. B. (1986) *Biochem. J.* **237**, 491–498
- Speiser, S. & Etlinger, J. D. (1982) *J. Biol. Chem.* **257**, 14122–14127
- Speiser, S. & Etlinger, J. D. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3577–3580
- Stacey, D. W. & Allfrey, V. G. (1977) *J. Cell Biol.* **75**, 807–817
- Tack, B. F., Dean, J., Eilat, D., Lorenz, P. E. & Schechter, A. N. (1980) *J. Biol. Chem.* **255**, 8842–8847
- Wharton, S. A. & Hipkiss, A. R. (1984) *FEBS Lett.* **184**, 249–253
- Zavortinck, M., Thacher, T. & Rechsteiner, M. (1979) *J. Cell. Physiol.* **100**, 175–186

Received 15 April 1986/12 September 1986; accepted 11 October 1986