Subcellular distribution of abnormal proteins in rabbit reticulocytes

Effects of cellular maturation, phenylhydrazine and inhibitors of ATP synthesis

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Inhibitors of ATP synthesis (cyanide, dinitrophenol, fluoride) inhibited proteolysis of pulse-labelled abnormal proteins in rabbit reticulocytes and caused an accumulation of the aberrant polypeptides in the cell debris fraction in a manner analogous to phenylhydrazine-induced Heinz bodies. When the reticulocytes were separated into age-groups by sedimentation through discontinuous gradients of bovine serum albumin, the ability of the cells to degrade puromycin peptides decreased with increasing cellular maturity and, in the more mature cells, up to 40% of the labelled abnormal polypeptide remained associated with the cell debris fraction at the end of the chase period. It is suggested that the association of the abnormal polypeptide with the cell debris fraction is a consequence of a maturation-induced loss, or an inhibitor-induced inactivation of the cellular proteolytic activity.

Reticulocyte *Proteolysis* *Puromycin* *Phenylhydrazine* *Heinz body*

1. INTRODUCTION

The ability to degrade selectively proteins whose structures deviate significantly from the normal gene products appears to be a widely distributed phenomenon in nature [1–8]. Synthesis of large quantities of abnormal protein in *Escherichia coli* results in its accumulation in so-called rapidly sedimenting complexes [6,7] which have been compared to Heinz bodies found in erythrocytes of humans suffering from certain haemoglobinopathies [9] and in rabbit reticulocytes after treatment with phenylhydrazine [10]. During maturation the rabbit reticulocyte loses much of its ability to degrade abnormal protein [5], which may explain the relatively infrequent occurrence of Heinz bodies in young reticulocytes [11], assuming that denatured haemoglobin and abnormal proteins are processed similarly. Here, the possibility that aberrant proteins are analogous to Heinz bodies is examined, and the effects of reticulocyte maturation and inhibition of ATP synthesis are also investigated.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

L-[U-^{14}C]Leucine (330 mCi/mmol) was obtain-

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ed from the Radiochemical Centre (Amersham), phenylhydrazine–HCl, puromycin dihydrochloride and *S*-2-aminoethyl-L-cysteine from Sigma (St Louis MO) and Sephadex G-100 from Pharmacia (Uppsala).

2.2. *Preparation, incubation and labelling of reticulocytes*

Rabbit reticulocytes [12] were labelled for 5 min with [^{14}C]leucine (1.5 μCi/ml cell suspension) as in [5]. Treatment with aminoethylcysteine and puromycin (5 and 25 μg/ml), and measurement of proteolysis during the chase period (the conversion of acid-precipitable radioactivity to acid-soluble following removal of the cells from excess isotope) were performed as in [5].

2.3. *Preparation of cell-free extracts and gel filtration*

Reticulocytes were lysed and cell-free supernatant fractions prepared (unless stated to the contrary) as in [13] and applied to a Sephadex G-100 column (85 cm × 2 cm diam.) and eluted with 0.1 M ammonium bicarbonate at 5°C.

2.4. *Assay of Heinz-body formation*

Heinz bodies were assayed as in [14]. The cells were incubated at 37°C and 50 μl samples were lysed and diluted with 3 ml 5 mM sodium-phosphate buffer (pH 7.4). The $A_{700\ nm}$ was measured before and after centrifugation at 600 × *g* for 5 min.

2.5. *Separation of reticulocytes into 10 cell age-groups*

Bovine serum albumin gradient fractionation of rabbit reticulocytes into 10 cell age groups was done as in [5]. After separation, the cells were washed, resuspended and pulse-labelled with [^{14}C]leucine and treated with 25 μg puromycin/ml as in section 2.2. At the start and after a 60 min chase period [5] cells were lysed [13] and processed as in section 3.

3. RESULTS

3.1. *Effect of cellular maturation on subcellular distribution of puromycin-peptides in rabbit reticulocytes*

The rate and extent of proteolysis of abnormal proteins in rabbit reticulocytes decrease dramatically during their maturation [5]. Whilst the drop in rate is explainable by decreased protease activity, the change in the final extent of proteolysis cannot be so simply understood. One explanation [5] is that some of the abnormal protein is converted into a form which is not degradable in the more mature cells and behaves like Heinz bodies in becoming sedimentable at low speeds. Age-fractionated reticulocytes were therefore pulse-labelled with leucine in the presence of 25 μg puromycin dihydrochloride/ml and then lysed at either the start or the end of the breakdown period (60 min). The broken cells were centrifuged at 2000 × *g* for 10 min, and the pellet obtained washed twice with the lysis buffer (the washings added to the supernatant fraction). The supernatant fraction was analysed by gel filtration through a Sephadex G-100 column in order to determine the proportion of the protein synthesised which was present as a high-M_r aggregate (void volume fraction) [13], which previous studies had shown to be a substrate for degradation [13]. Both the rate of proteolysis and its maximum extent decrease during cell maturation (fig.1; [5]). The proportion of the radioactivity initially found in the high-M_r fraction also decreased with cellular maturity and less of that which was formed was catabolised in the older cells. The radioactivity in the 2000 × *g* pellet fraction was greater in the more mature cells; only the 3 youngest cell age groups appeared to degrade the 2000 × *g* pelletable material.

3.2. *Effect of inhibitors of ATP synthesis on the subcellular distribution of aberrant proteins in rabbit reticulocytes*

Proteolysis of certain abnormal proteins in reticulocytes is ATP-dependent [3,4]. Addition of cyanide, fluoride and dinitrophenol significantly inhibits degradation of aberrant protein synthesised in response to the lysine analogue aminoethylcysteine [15] (and table 1). In the inhibited cells labelled abnormal protein accumulated in the 600 × *g* pellet (cell debris) fraction although the loss of the labelled protein from the Sephadex G-100 void volume fraction during the chase period also appears to be somewhat inhibited [15]. Using puromycin at 25 μg/ml to promote the synthesis of abnormal protein of shortened chain length both effects were decreased (table 1).

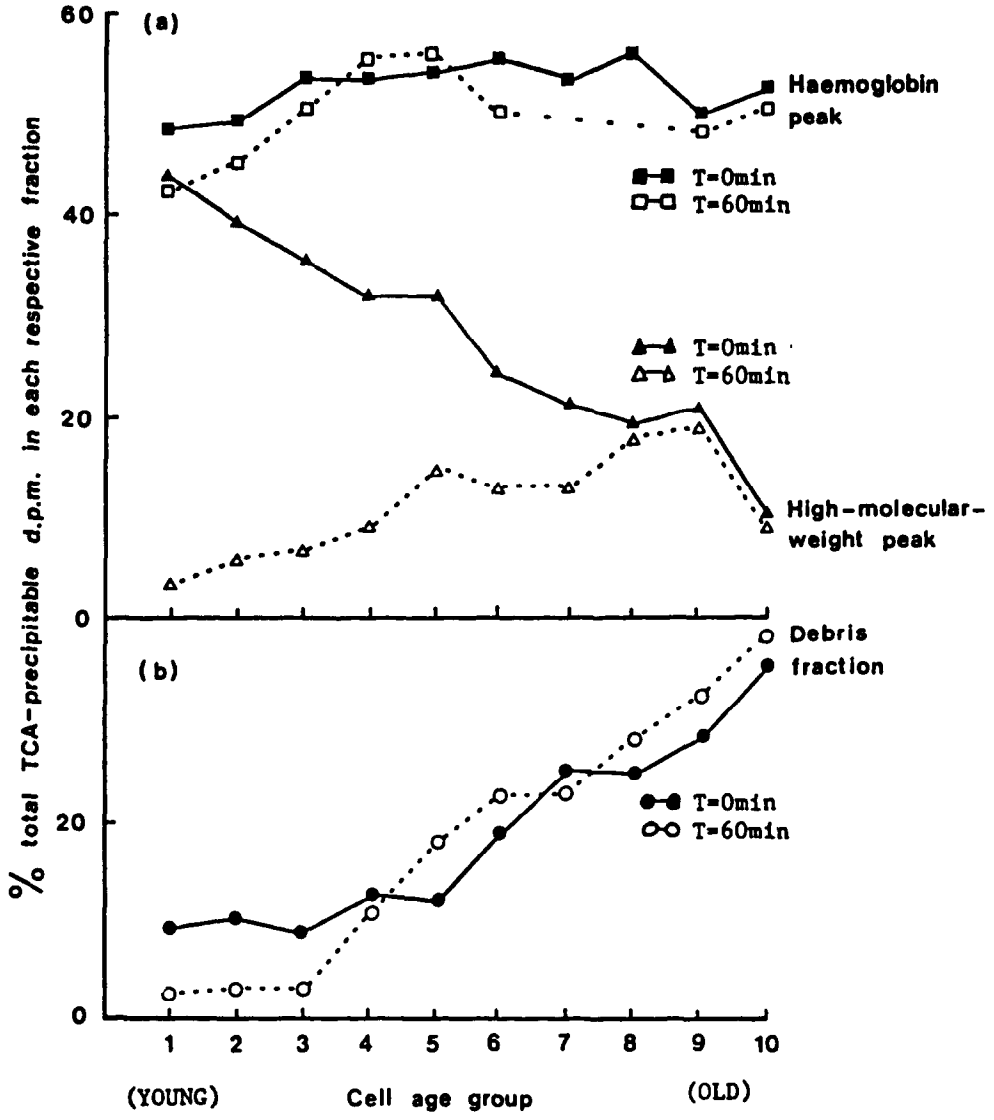

Fig.1. Effect of reticulocyte maturation on the subcellular distribution of puromycin peptides. Age-separated rabbit reticulocytes were pulse-labelled with [^{14}C]leucine in the presence of 25 μg puromycin dihydrochloride/ml. The cells were lysed at the start (T = 0) or at the end (T = 60 min) of the chase period. The 2000 × g supernatant fractions were chromatographed on a Sephadex G-100 column and the trichloroacetic acid-precipitable radioactivity which eluted in the void volume region (high-M_r peak) and with the haemoglobin were recorded (a). Panel (b) shows the trichloroacetic acid-precipitable radioactivity which remained with the cell-debris fraction (2000 × g pellet) (see text for details). Percentage proteolysis in each cell age group was as follows: (1) 65.7, (2) 49.7, (3) 39.9, (4) 25.1, (5) 18.4, (6) 16.7, (7) 15.2, (8) 14.9, (9) 6.3, (10) 1.6.

3.3. *Effects of phenylhydrazine on proteolysis and subcellular distribution of aberrant proteins in rabbit reticulocytes*

Phenylhydrazine-treatment of erythrocytes promotes the formation of Heinz-bodies resulting in an increase in sedimentable turbidity after cell lysis [14]. Fig.2 shows that rabbit reticulocytes respond similarly: the amount of sedimentable turbidity increases with phenylhydrazine concentration and duration of treatment. Addition of 1 mM

Table 1

The effects of fluoride, dinitrophenol, cyanide and phenylhydrazine on the subcellular distribution of pulse-labelled aberrant polypeptide in rabbit reticulocytes

Protein degraded Period of proteolysis Inhibitor added during chase	Cellular radioactivity (%) in:		Proteolysis (%)
	Cell debris fraction	High-M_r fraction	
Puromycin-peptides (25 μg puromycin/ml)			
0 min	45.6	16.2	0
60 min	15.0	1.2	77.2
+ fluoride	22.4	1.5	68.0
+ DNP	18.4	1.5	73.7
+ cyanide	28.4	2.3	57.4
+ phenylhydrazine	18.1	1.3	74.7
Puromycin-peptides (5 μg puromycin/ml)			
0 min	25.6	17.9	0
60 min	10.1	1.6	48.5
+ fluoride	21.6	4.1	32.5
+ DNP	20.5	3.9	32.2
+ cyanide	33.0	6.4	17.3
+ phenylhydrazine	22.1	3.7	24.5
Aminoethylcysteine-protein			
0 min	26.2	16.4	0
60 min	9.1	2.9	42.6
+ fluoride	30.2	6.3	18.0
+ DNP	31.1	4.0	33.1
+ cyanide	40.3	7.0	10.8
+ phenylhydrazine	34.9	4.5	17.2
Normal protein			
0 min	15.9	4.1	0
60 min	7.8	2.2	3.6
+ fluoride	17.3	4.6	0.8
+ DNP	9.8	4.5	2.1
+ cyanide	26.4	3.9	0.7
+ phenylhydrazine	27.4	5.6	5.7

Rabbit reticulocytes were pulse-labelled with [^{14}C]leucine in the presence of aminoethylcysteine, or puromycin (5 or 25 μg/ml). The proportion of cellular trichloroacetic acid-precipitable radioactivity which was present in the cell debris fraction (600 × *g* pellet fraction) and in the void volume region (termed high-M_r aggregate) following Sephadex G-100 chromatography of the 600 × *g* supernatant fraction, was determined. Inhibitors were added at the start of the chase period; sodium cyanide, sodium fluoride, and dinitrophenol were employed at 50 mM, and phenylhydrazine added to 1 mM

phenylhydrazine during the chase period to cells containing only normal proteins promotes the accumulation of radioactivity in the 600 × *g* pellet fraction (table 1). The effect of phenylhydrazine on the cells which contained labelled aberrant protein varied; an accumulation of radioactivity in the 600 × *g* pellet fraction was observed in the case of the aminoethylcysteine-treated cells; in puromycin-treated cells, this effect decreased with increasing antibiotic concentration. Phenylhydrazine stimulated (by 58%) proteolysis in control cells (table 1) which suggests that at least some of the protein (presumably haemoglobin) becomes susceptible to the reticulocyte protease(s) in the presence of phenylhydrazine. In contrast, phenylhydrazine inhibited breakdown of abnormal proteins by 59.6%, 49.5% and 3.2% respectively in aminoethylcysteine, 5 μg/ml and 25 μg/ml puromycin-treated cells. These effects can be explained by the suggestion that phenylhydrazine affects all haemoglobin molecules present (most of which will be non-radioactive) so that in cells containing labelled normal protein denaturation to proteolytic susceptibility is reflected by an increase in the amount of radioactive protein degraded. However, in cells which had synthesised aminoethylcysteine-induced abnormal protein of high specific radioactivity, phenylhydrazine-denatured non-radioactive haemoglobin would act as an alternative substrate for the cellular protease(s) and saturate the degradative process to bring about a decrease in the catabolism of labelled aberrant protein. In puromycin-treated cells, the proteins synthesised are of shorter chain length than either phenylhydrazine-denatured or aminoethylcysteine-substituted globin [15] and may enter the degradative pathway 'downstream' from the initial catabolic event in the degradation of normal length globin chains [15].

Heat instability is a simple test for the presence of altered and unstable haemoglobin in erythrocytes [16]. Heat treatment of rabbit reticulocyte lysates derived from cells labelled in

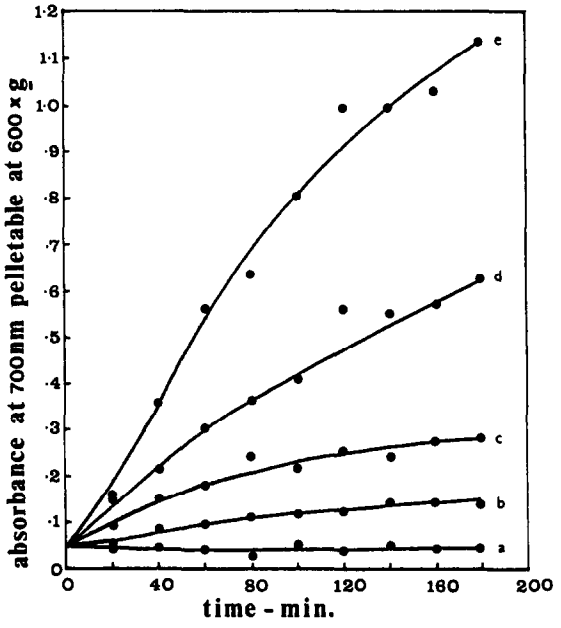

Fig.2. The effect of phenylhydrazine on Heinz-body formation in rabbit reticulocytes. Reticulocytes were incubated at 37°C in phosphate-buffered saline [5] and phenylhydrazine added at zero time to give final concentrations of: (a) 0, (b) 0.5, (c) 1.0, (d) 2.5, (e) 5.0 mM. Heinz-body formation was detected as an increase in sedimentable turbidity after cell lysis [14].

the presence of either lysine or aminoethylcysteine showed that there was more heat-unstable radioactive protein in lysates derived from analogue-treated cells than in lysates derived from control cells (22% and 7%, respectively). Addition of phenylhydrazine to the cell lysates prior to heat treatment caused a dramatic increase in the proportion of protein precipitated in both cell-lysates. Again more of the radioactive analogue-containing protein was precipitated, compared to radioactive lysine-containing protein from control cells (90% and 79%, respectively). These observations are consistent with those in [17] where analogue-substituted rabbit haemoglobin was prone to isopropanol-induced denaturation [17].

4. DISCUSSION

These observations indicate that, should the reticulocyte degradative system become inactive (by inhibitors of ATP synthesis) or saturated by excess substrate following the addition of phenylhydrazine, the labelled aberrant protein accumulates in the cell debris fraction in a manner analogous to Heinz bodies. Because the reticulocyte loses protease [5] and peptidase [18] activity as it matures, the degradative apparatus is likely to become more readily saturable as the cell matures, which may explain the increased occurrence of the Heinz-body-like material in the more mature cells. Addition of the protease inhibitor *N*-tosyl-L-phenylalanylchloromethyl ketone also promoted the accumulation of aberrant polypeptide in the cell-debris fraction of amino acid analogue-treated rabbit reticulocytes [17].

An increased incidence in the formation of Heinz-bodies is associated with the maturation of human erythroid cells [11]. During β-thalassaemic erythroid cell maturation, the bone marrow erythroid cells of increasing maturity display decreased ability to degrade excess α-globin chains [19] and the excess α-globin chains are associated with the formation of Heinz-bodies.

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