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TRANSFERRIN UPTAKE AND RELEASE BY RETICULOCYTES TREATED WITH PROTEOLYTIC ENZYMES AND NEURAMINIDASE

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SUMMARY

The mechanism of transferrin uptake by reticulocytes was investigated using rabbit transferrin labelled with ^{125}I and ^{59}Fe and rabbit reticulocytes which had been treated with trypsin, Pronase or neuraminidase. Low concentrations of the proteolytic enzymes produced a small increase in transferrin and iron uptake by the cells. However, higher concentrations or incubation of the cells with the enzymes for longer periods caused a marked fall in transferrin and iron uptake. This fall was associated with a reduction in the proportion of cellular transferrin which was bound to a cell membrane component solubilized with the non-ionic detergent, Teric 12A9. The effect of trypsin and Pronase on transferrin release from the cells was investigated in the absence and in the presence of *N*-ethylmaleimide which inhibits the normal process of transferrin release. It was found that only a small proportion of transferrin which had been taken up by reticulocytes at 37 °C but nearly all that taken up 4 °C was released when the cells were subsequently incubated with trypsin plus *N*-ethylmaleimide, despite the fact that about 80 % of the ^{59}Fe in the cells was released in both instances. Neuraminidase produced no change in transferrin and iron uptake by the cells.

These experiments provide evidence that transferrin uptake by reticulocytes requires interaction with a receptor which is protein in nature and that following uptake at 37 °C, most of the transferrin is located at a site unavailable to the action of proteolytic enzymes. The results support the hypothesis that transferrin enters reticulocytes by endocytosis.

INTRODUCTION

Immature erythropoietic cells including reticulocytes are able to remove iron bound to its plasma-carrying protein, transferrin, and utilize it for haemoglobin synthesis [1, 2]. This process involves an interaction between the transferrin molecule and the cell surface [3, 4] which occurs in at least two stages, an initial rapid and temperature-insensitive adsorption reaction followed by a slower, temperature-sensitive stage of progressive uptake [5, 6]. Subsequently, the iron is removed from

the transferrin and the protein is released from the cell [3, 4]. It is believed that the uptake of transferrin by the cell involves an interaction at specific membrane receptors [3], but the nature of these receptors and the mechanism of their reaction with transferrin is poorly understood. There is evidence that the transferrin molecules taken up by reticulocytes pass through the outer cell membrane to the interior of the cell [7, 8]. If so, it is likely that the receptors play a part in the process of transport across the cell membrane. This would be an example of an important function of a specific receptor in addition to that of simply reacting with its ligand. However, the concept that transferrin enters cells as part of its iron-donating function is disputed by some workers [9] and hence requires further investigation.

The mechanism of the interaction between transferrin and its receptors, and the question of whether transferrin passes into cells are important problems which must be solved before a proper understanding of the mechanism of iron assimilation by cells will be achieved. The aim of the present work was to investigate these problems by studying the effects of treatment of reticulocytes with proteolytic enzymes and neuraminidase on the processes of uptake and release of transferrin.

MATERIALS AND METHODS

Chemicals. Trypsin (from bovine pancreas, Type III) and *N*-ethylmaleimide (MalNEt) were from Sigma Chemical Co., St. Louis, Mo., U.S.A., and neuraminidase (from *Vibrio cholerae*, 500 units/ml) was obtained from BDH Chemicals Ltd., Poole, U.K. Pronase (B Grade), trypsin inhibitor (from egg white, B Grade) and Hide Powder Azure (B Grade) were purchased from Calbiochem, San Diego, Calif., U.S.A. The non-ionic detergent Teric 12A9, lauryl alcohol condensed with ethylene oxide, was a gift from I.C.I. Research Laboratories, Cheshire, U.K. The radioisotopes ^{59}Fe ($FeCl_3$, spec. act. 10–30 Ci/g Fe) and ^{125}I and ^{131}I (NaI, carrier free) were obtained from The Radiochemical Centre, Amersham, U.K.

Rabbit reticulocytes. Induction of reticulocytosis in rabbits by repeated bleeding or by the injection of phenylhydrazine and the method of washing the cells has been described previously [10, 11]. The buffy layer was aspirated after each wash.

Purification and labelling of proteins. Rabbit transferrin and albumin were isolated as in earlier investigations [12, 13] and labelled with ^{125}I and ^{131}I by the iodine monochloride method [14]. Apotransferrin or fresh rabbit plasma were labelled with ^{59}Fe by addition of the ^{59}Fe as its complex with nitrilotriacetic acid used at twice the molar concentration of iron, followed by addition of $NaHCO_3$ to give a final HCO_3^- concentration of 20 mM and incubation at 37 °C for 1 h. The amount of iron added to the transferrin solutions was always less than that required to raise the degree of saturation of the transferrin with iron to 50 %.

Transferrin and iron-uptake experiments. The enzymes were dissolved in a buffer solution consisting of equal volumes of Hanks and Wallace balanced-salt solution [15] and phosphate-buffered saline (1 vol. 310 mOsm sodium phosphate, pH 7.4 and 19 vol. 0.15 M NaCl). Washed reticulocytes were preincubated with a freshly prepared enzyme solution at 37 °C with constant shaking for the required period of time. The cells were then washed three times with ice-cold 0.15 M NaCl to remove the enzyme before use in the uptake studies. Control samples of cells were incubated and washed in the same way but in the absence of the enzymes. When cell

extracts were incubated with trypsin the digestion of the extract was inhibited after the preincubation period by the addition of trypsin inhibitor at twice the amount required to inhibit the amount of trypsin used, according to the specifications of the supplier of the inhibitor. In control experiments it was shown that the inhibitor, when used in this way, prevented any further change in the capacity of cells, membranes or extracts to take up transferrin or iron, and that the inhibitor by itself or inhibitor plus trypsin did not block transferrin or iron uptake.

Unless otherwise stated the procedures used in the present investigation for incubating the cells with labelled proteins, washing the cells and measuring radioactivity were as described in earlier publications [6, 10].

Transferrin and iron-release experiments. To measure the release of transferrin and iron from reticulocytes the cells were first incubated with transferrin labelled with ^{125}I and ^{59}Fe. They were then washed three times with ice-cold 0.15 M NaCl and reincubated at 37 °C in 4 vol. Hanks and Wallace solution. At different time intervals, samples of the cell suspension were transferred to 3 ml ice-cold 0.15 M NaCl, centrifuged at $2000 \times g$ for 30 min at 4 °C and radioactivity counted separately in the supernatant solution and the cells.

Preparation of ghosts and extraction with Teric 12A9. Unsealed reticulocyte ghosts were prepared from washed cells by the method of Dodge *et al.* [16]. The washed ghosts were extracted with 4 vol. ice-cold 1 % Teric in 20 mosm sodium phosphate buffer, pH 7.4 for 30 min and were then centrifuged at $40\,000 \times g$ for 30 min to separate residue and solubilized extract. Sealed ghosts were prepared as in earlier work [17].

Electron microscopy and autoradiography. Previously described methods were used for the preparation and examinations of reticulocytes by transmission electron microscopy and electron microscope autoradiography [7] and for scanning electron microscopy [18].

Analytical methods. New methylene blue was used to determine reticulocyte counts. Haemoglobin was measured as oxyhaemoglobin. Haem was extracted from cells according to Thunell [19]. Proteolytic activity was measured by two procedures, the X-ray film digestion method [20] and the Remazol/brilliant Blue/Hide technique [21]. Sialic acid was determined by Warren's method [22]. Iron was determined by the procedure recommended by the International Committee for Standardization in Haematology [23].

RESULTS

Effect of proteolytic enzymes on transferrin and iron uptake

Varying enzyme concentration. The patterns of transferrin and iron uptake observed with control reticulocytes and with reticulocytes preincubated for 10 min at 37 °C with Pronase at concentrations of 2 μg/ml and 400 μg/ml are shown in Fig. 1. Transferrin uptake occurred in three phases which have been described previously [6]. The first is adsorption which is represented by the amount of transferrin bound by the cells immediately on mixing with the transferrin (zero time in Fig. 1). This is followed by the progressive uptake and plateau phases which occur during subsequent incubation at 37 °C. Iron uptake was linear during the period of incubation and could be described by a single rate value. The low concentration of Pronase (2 μg/ml)

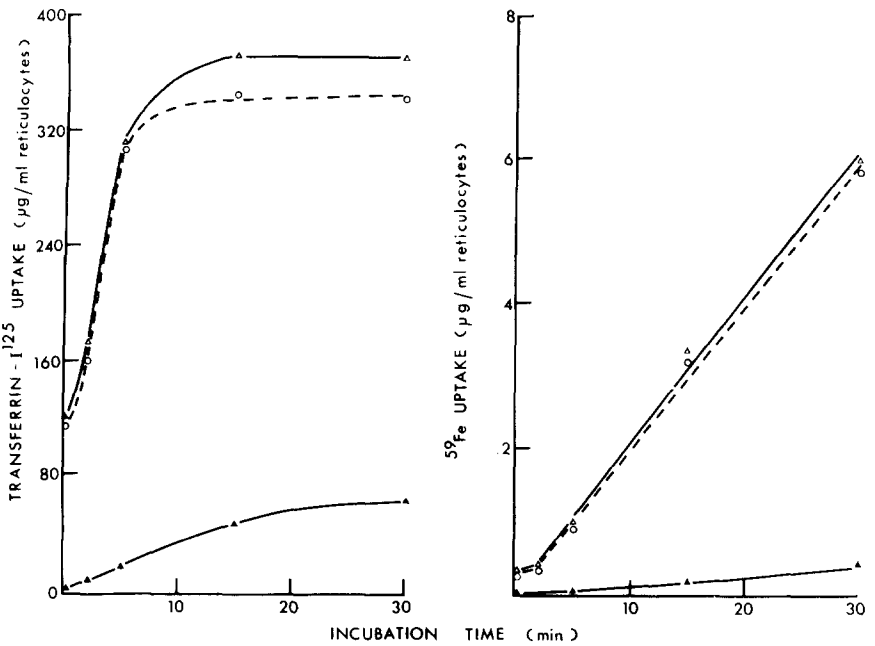

Fig. 1. Effect of incubation of rabbit reticulocytes with Pronase on subsequent uptake of transferrin and iron. The cells (35 % reticulocytes) were incubated for 10 min at 37 °C with no Pronase (○ ---○) or with Pronase at concentrations of 2 μg per ml (△—△) or 400 μg per ml (▲—▲). They were then washed three times and reincubated with transferrin labelled with ^{125}I and ^{59}Fe (transferrin concentration, 3.10 mg per ml; iron concentration, 1.50 μg per ml).

produced a small but reproducible increase in the amount of transferrin adsorbed by the cells and the total amount taken up when the plateau phase was reached plus an increase in the rate of iron uptake. At a concentration of 400 μg/ml Pronase produced a marked inhibition of transferrin adsorption, rate of progressive uptake and total amount taken up and in the rate of iron uptake.

The effects of 10 min pretreatment of the cells with varying concentrations of Pronase and of trypsin are shown in Table I. Trypsin in a concentration of 4 μg/ml produced an enhanced amount of adsorption and total uptake of transferrin and increased rate of iron uptake, similar to that found with Pronase at 2 μg/ml (Fig. 1). This enhancement of transferrin and iron uptake by reticulocytes incubated with low concentrations of trypsin or Pronase was found in four separate experiments. Treatment with increasing concentrations of the enzymes progressively produced greater degrees of inhibition of all three phases of transferrin uptake and of the rate of iron uptake. However, the proportion of the ^{59}Fe taken up by the cells which was incorporated into haem was markedly elevated at the higher enzyme concentrations. The uptake of albumin by the cells also increased slightly.

Varying time of incubation with the enzymes. Pretreatment of the cells with trypsin (100 μg/ml) or Pronase (50 μg/ml) for increasing periods of time up to 60 min led to a progressive inhibition of transferrin and iron uptake. After 60 min incubation with either enzyme the rate of both transferrin and iron uptake were reduced to less than 10 % of the control values. The percent incorporation of ^{59}Fe into haem in-

TABLE I

EFFECT OF TRYPSIN AND PRONASE ON IRON AND TRANSFERRIN UPTAKE BY RABBIT RETICULOCYTES

Reticulocytes were preincubated with the enzymes at 37 °C for 10 min, washed three times and then incubated with transferrin labelled with ^{125}I and ^{59}Fe. Control cells were preincubated in the absence of the enzymes. Results for transferrin are given as adsorption, the amount bound by the cells immediately on mixing in the cold with labelled transferrin; rate, the initial rate of uptake of transferrin during the first two min of incubation at 37 °C; amount, the total amount of transferrin bound by the cells after incubation for 30 min at 37 °C. The haem values refer to the percentage of total ^{59}Fe taken up by the cells which was incorporated into haem after 30 min incubation. The reticulocyte count was 25 %, iron concentration 2.9 μg per ml and transferrin concentration 3.3 mg per ml.

Enzyme	Concentration of enzyme (μg/ml)	Uptake (% control value)					
		Transferrin			Iron		Albumin
		Adsorption	Rate	Amount	Rate	Haem	Amount
Trypsin	4	105	103	104	110	86	95
	10	79	95	104	100	92	100
	50	83	90	91	100	–	–
	100	66	89	92	90	131	106
	200	44	79	91	80	–	–
	400	31	64	75	66	135	111
Pronase	4	99	98	106	107	99	111
	10	89	93	105	109	96	108
	50	38	92	107	89	–	–
	100	15	45	62	44	164	116
	200	5	18	35	17	–	–
	400	3	8	19	7	168	113

creased as the rate of iron uptake decreased so that by 60 min it was approximately twice as great in the enzyme-treated cells as in the controls (94 % compared with 45 %).

Recovery of function of enzyme-treated cells. Reticulocytes were incubated with trypsin or Pronase, washed (and trypsin inhibited with its inhibitor) and reincubated in Hanks and Wallace solution at 37 °C for 1 and 3 h before measuring transferrin and iron uptake. However, no recovery of function was observed.

Morphology of enzyme-treated cells. Reticulocytes which had been treated with trypsin (100 μg/ml) or Pronase (50 μg/ml) at 37 ° for 10 min were examined by both transmission and scanning electron microscopy. No differences between treated and control cells was observed.

Extraction of reticulocytes with Teric 12A9. When untreated reticulocytes were incubated with ^{125}I-labelled transferrin and ^{131}I-labelled albumin, washed, extracted with Teric 12A9 and the supernatant solution obtained after centrifugation was fractionated by gel filtration on Sephadex G-200 the transferrin radioactivity was eluted in two peaks (Fig. 2A). The first peak emerged just after the void volume of the column and the second peak at a volume corresponding to that of ^{125}I-labelled transferrin alone. This double-peak type of elution profile for ^{125}I-labelled transferrin was also demonstrable in two other ways, viz. (1) incubation of reticulocyte ghosts with ^{125}I-labelled transferrin and ^{131}I-labelled albumin followed by washing

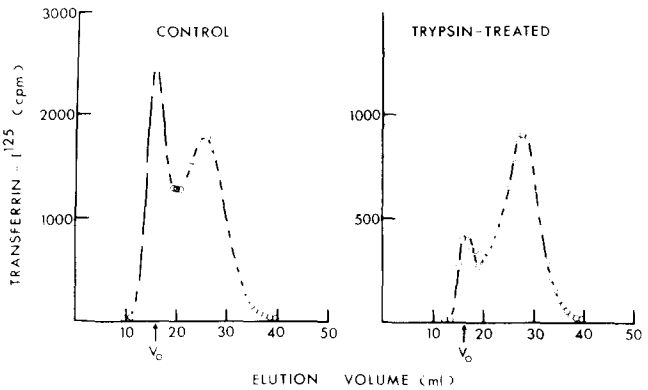

Fig. 2. Sephadex G-200 gel filtration of Teric 12A9 extracts of ghosts prepared from control and trypsin-treated reticulocytes which had been incubated with ^{125}I- and ^{59}Fe-labelled transferrin. Rabbit blood cells (63 % reticulocytes) were incubated for 10 min at 37 °C in the absence of trypsin (control) or with trypsin (1.6 mg per ml). The cells were then washed three times, reincubated with the labelled iron-saturated transferrin (1 mg per ml), rewashed, haemolysed and the ghosts washed and extracted with Teric 12A9 as described in the text.

of the ghosts, extraction with Teric and gel filtration, and (2) incubation of a Teric extract of reticulocytes with ^{125}I-labelled transferrin and ^{131}I-labelled albumin for 30 min at 37 °C immediately before gel filtration. By contrast, ^{131}I-labelled albumin eluted as only one peak near that expected of the protein alone. Trypsin treatment of intact reticulocytes, reticulocyte ghosts or a Teric extract of the ghosts prior to incubation with ^{125}I-labelled transferrin led to a marked reduction in the relative size of the first elution peak found on gel filtration. This is illustrated for whole cells in Fig. 2B. A similar degree of reduction in the relative size of the first peak was obtained when ghosts or Teric extracts were treated with trypsin before incubation with labelled transferrin. The first peak could also be reduced greatly by incubating ghosts or Teric extracts with unlabelled transferrin before incubation with ^{125}I-labelled transferrin but not by incubation with albumin.

The mode of action of trypsin on the process of transferrin uptake by reticulocytes was investigated in another experiment in which Teric extracts were used. Sealed ghosts prepared from reticulocytes or equivalent quantities of intact reticulocytes were incubated in 0.15 M NaCl with or without the addition of trypsin (0.8 mg/ml), washed and Teric extracts were prepared from the ghosts or from the cells after preparation of ghosts. The extracts were incubated with ^{125}I-labelled transferrin and fractionated by gel filtration. As shown in Fig. 3, trypsin treatment of intact reticulocytes caused a much greater reduction in the relative size of the first ^{125}I-labelled transferrin peak than was found with the sealed ghosts.

Thus, trypsin treatment of intact reticulocytes caused a reduction in the percent of total ^{125}I labelled transferrin present in the first peak from 27 to 12. However, with trypsin treatment of ghosts the percent ^{125}I-labelled transferrin in the first peak only fell from 17 to 13.

Effect of proteolytic enzymes on transferrin and iron release from reticulocytes

Investigations were made on the effects of trypsin and Pronase on transferrin

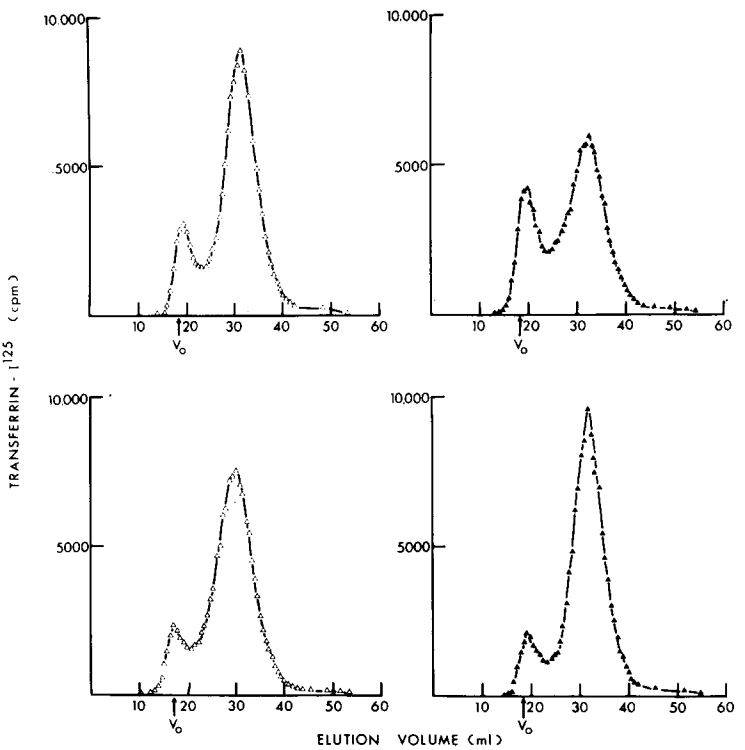

Fig. 3. Sephadex G-200 gel filtration of Teric 12A9 extracts of ghosts obtained from rabbit reticulocytes which had been incubated with trypsin while in the form of intact cells or after preparation of released ghosts. Resealed ghosts (△—△) from reticulocyte-rich blood (80 % reticulocytes) or intact cells (▲—▲) were incubated with trypsin plus trypsin inhibitor (A, B) or with trypsin alone (C, D) for 10 min at 37 °C. Trypsin inhibitor was then added to C and D. All incubations were continued for a further 5 min. The ghosts and cells were then washed, Teric extracts prepared from the ghosts or from the cells after preparation of ghosts and the extracts were incubated with ^{125}I-labelled transferrin for 15 min at 37 °C before fractionation by gel filtration. The concentration of trypsin used was 0.8 mg per ml and the amount of ^{125}I-labelled transferrin added to the Teric extracts was 0.34 μg per ml reticulocytes from which the extracts were prepared.

and iron release by cells which had taken up ^{59}Fe and ^{125}I-labelled transferrin during incubation for 15 min at two incubation temperatures, 4 or 37 °C. The effect of 5 mM MalNEt on the release process was also studied both in the presence and absence of the enzymes. It has been shown previously that MalNEt almost completely inhibits transferrin release from reticulocytes [10]. The results obtained with trypsin and pronase were very similar, hence only those obtained with trypsin will be presented.

The pattern of release of ^{125}I and ^{59}Fe from reticulocytes which had taken up the doubly labelled transferrin at 4 °C was different from that obtained following uptake at 37 °C (Fig. 4). With the 4 °C uptake, nearly half of the ^{125}I on the cells was released immediately on commencing reincubation at 37 °C. After this rapid release the amount of ^{125}I in the medium decreased for about 3 min and then increased to reach a plateau level after about 60 min. With the 37 °C uptake, ^{125}I release was initially low, but increased rapidly to reach approximately the same plateau

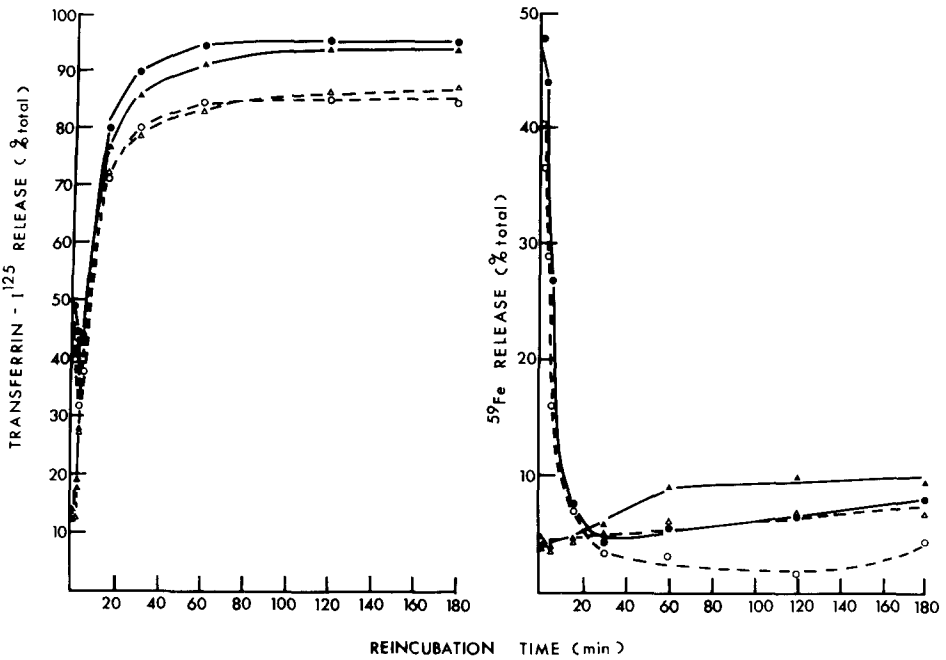

Fig. 4. Effect of incubation with trypsin on the release of ^{125}I-labelled transferrin and ^{59}Fe from rabbit reticulocytes. The cells (20 % reticulocytes) were incubated with transferrin labelled with ^{125}I and ^{59}Fe for 15 min at 4 °C (○, ●) or 37 °C (△, ▲). They were then washed with ice-cold 0.15 M NaCl and reincubated at 37 °C in Hanks and Wallace solution in the absence (△, ○) or in the presence of trypsin (▲, ●) in a concentration of 0.33 mg per ml.

level after 60 min. The effect of adding trypsin to the incubation medium during the period of release was to increase the amount of ^{125}I released at the plateau by about 10 % (Fig. 4).

As was found with ^{125}I, nearly 50 % of the ^{59}Fe taken up at 4 °C was released immediately after commencing reincubation but the ^{59}Fe in the medium then fell during the next 15–30 min to reach a level equivalent to between 2 and 4 % of that in the cells. The release of ^{59}Fe from cells which had taken it up at 37 °C was low, rising from 4 % at 0 min to 7 % after 180 min reincubation. Treatment with trypsin had no significant effect on the release of ^{59}Fe during the first 30–60 min of reincubation but thereafter caused approximately a 5 % increase whether or not uptake had occurred at 4 or 37 °C. This increase was probably due to the release of ^{59}Fe-labelled haemoglobin from the cells since a small degree of haemolysis was observed when the cells were incubated for 1–3 h in the presence of trypsin.

The addition of MalNEt to the reincubation solution effectively blocked the release of ^{125}I-labelled transferrin from reticulocytes, whether the protein had been taken up at 4 or at 37 °C (Fig. 5). In the presence of both trypsin and MalNEt, the amount of ^{125}I released from the cells which had taken up the labelled protein at 4 °C (Fig. 5) was approx. 85 % of that released in the presence of trypsin alone (Fig. 4). This ability of trypsin to overcome the inhibitory effect of MalNEt on transferrin release was much less marked when the cells were allowed to take up the transferrin at

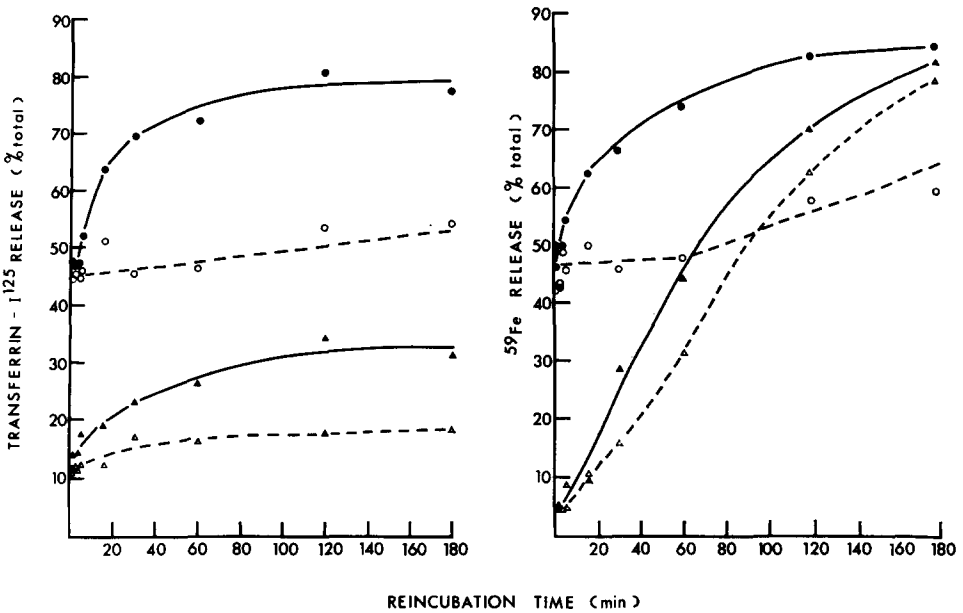

Fig. 5. Effect of incubation with MalNEt and trypsin on the release of ^{125}I-labelled transferrin and ^{59}Fe from rabbit reticulocytes. The cells (20 % reticulocytes) were incubated with transferrin labelled with ^{125}I and ^{59}Fe for 15 min at 4 °C (○, ●) or 37 °C (△, ▲). They were then washed with ice-cold 0.15 M NaCl, MalNEt (5 mM) was added and the cells maintained at 4 °C for 5 min before reincubation in Hanks and Wallace solution at 37 °C was performed in the absence (△, ○) or in the presence of trypsin (▲, ●) in a concentration of 0.33 mg per ml.

37 °C. In the latter case, when the reincubation solution contained both trypsin and MalNEt, the amount of ^{125}I which appeared in the solution during reincubation (Fig. 5) was only 35 % of that found when the solution contained only trypsin and no sulfhydryl-reacting agent (Fig. 4).

The release of ^{59}Fe from the cells was quite different in the presence of MalNEt (Fig. 5) than in its absence (Fig. 4). Following uptake at 4 °C the effect of MalNEt was to inhibit any change in the amount of ^{59}Fe present in the incubation medium during the first 60 min of reincubation. This was followed by a small increase in the amount of ^{59}Fe released during the subsequent 2 h. When trypsin and MalNEt were combined, a substantial amount of ^{59}Fe was released from the cells. The pattern of ^{59}Fe release from the reticulocytes treated with MalNEt closely parallelled that of ^{125}I release both in the presence and absence of trypsin when the initial uptake had taken place at 4 °C. However, this was not the case following 37 °C uptake. In this situation, reincubation with MalNEt was accompanied by a progressive release of ^{59}Fe and haemoglobin from the cells, only slightly greater in the presence of trypsin, so that after a 3-h reincubation, approx. 80 % of the ^{59}Fe and a similar percentage of the haemoglobin had been released from the cells.

Autoradiography of cell samples reincubated for 1.5 h in the presence of MalNEt showed that silver grains, presumably representing ^{125}I-labelled transferrin molecules, were present on the periphery and inside both intact and haemolysed cells (Fig. 6).

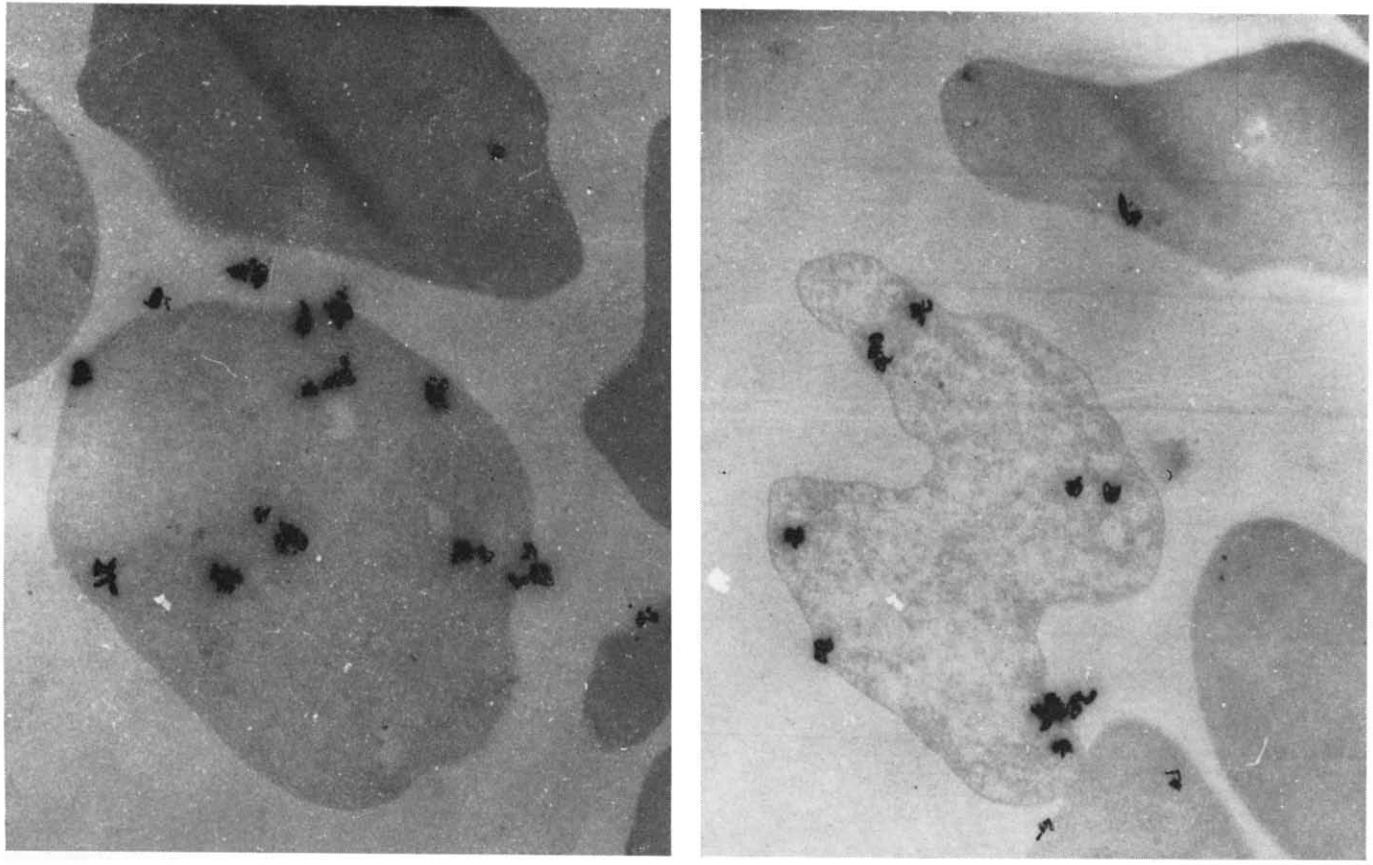

Fig. 6. Autoradiographs of reticulocytes after incubation with ^{125}I-labelled for 15 min followed by washing and reincubation for 1.5 h in the presence of MalNEt (5 mM). The photograph on the left shows intact cells, that on the right shows a haemolysed cell and parts of intact cells. Magnification: × 16 500.

In control experiments it was shown that the proteolytic activity of trypsin and pronase were unimpaired by the presence of 5 mM MalNEt, using two methods of measuring proteolytic activity, X-ray film digestion and the Remazol/brilliant blue/Hide technique.

Effect of neuraminidase on transferrin and iron uptake by reticulocytes

Treatment of the cells with neuraminidase at 10–60 units/ml for 45 min so as to release about 30–50 % of the plasma membrane sialic acid had no effect on the rate of iron uptake or any aspect of transferrin uptake by reticulocytes. The incorporation of the iron taken up into haem was also unimpaired. Treatment of intact reticulocytes, their ghosts or a Teric extract of reticulocytes with neuraminidase did not affect the gel filtration-elution pattern obtained when cells, ghosts or extract were incubated with ^{125}I-labelled transferrin before gel filtration.

DISCUSSION

The results of the present investigations of the effects of proteolytic enzymes on transferrin and iron uptake by reticulocytes confirm those of earlier work [2, 3] and extend them by providing information on the mechanisms involved. The enzymes could have exerted their effects by acting on the transferrin receptors or on other membrane proteins or glycoproteins so as to cause steric or charge effects within the membrane which could inhibit transferrin and iron uptake. A direct effect on the receptors is the most likely explanation. Thus, the enzymes inhibited transferrin but not albumin adsorption to the cells. Furthermore, the studies in which the membranes were extracted with Teric 12A9 showed that the enzymes altered a component of the membrane extract so that it no longer interacted with transferrin. The observations that albumin did not react with this component and that ^{125}I-labelled transferrin was displaced from the complex with it by incubation with unlabelled transferrin suggest that the component is the transferrin receptor. Fielding and Speyer [24] have shown that part of the transferrin extracted from reticulocytes with Triton X-100 is in the form of a complex with a membrane component which they believe to be the transferrin receptor.

The action of the proteolytic enzymes on reticulocytes seemed to be confined to the membranes. Thus, the intracellular metabolism of iron taken up by the cells was not impaired. Indeed, the efficiency of incorporation of ^{59}Fe into haem increased when the rate of iron uptake was low, presumably because at normal rates of uptake iron enters the cell more quickly than it can be incorporated into haem.

The degree of proteolytic digestion of the membranes required to inhibit transferrin and iron uptake appears to be relatively small since cells treated to produce these changes in function showed no evidence of haemolysis or of morphological changes when examined by electron microscopy. Even so, the changes were not reversed during a 3-h reincubation in the absence of the enzymes. This suggests that reticulocytes may not be able to synthesize transferrin receptors, although a more detailed investigation would be required to prove this point.

Incubation of reticulocytes with low concentrations of proteolytic enzymes or for short periods of time enhanced the uptake of transferrin and iron (Table I). It is possible that some of the transferrin receptors were masked by other membrane

components which could be removed by brief treatment with the enzymes making the receptors accessible to transferrin. A similar increase of protein binding by the membrane has been reported for fat cells which were found to bind more insulin after brief treatment with phospholipase A or C [25].

The results obtained in the experiments in which the release of transferrin and iron from reticulocytes was measured support the concept that transferrin molecules pass through or at least deeply into the cell membrane during the second, temperature-sensitive phase of transferrin uptake. It was found that in the presence of both MalNEt and trypsin, transferrin was largely released from the cells if it had been taken up at 4 °C but little was released if taken up at 37 °C (Fig. 5). Thus, after uptake at 4 °C the ^{125}I-labelled transferrin was susceptible to release by proteolytic digestion, indicating that it was at sites on the surface of the cell which could be reached by the enzymes. This agrees with the concept that after incubation of ^{125}I-labelled transferrin and reticulocytes at 4 °C the transferrin molecules are bound to membrane-binding sites (receptors) that are readily released by proteolytic digestion. After uptake of the ^{125}I-labelled transferrin at 37 °C, however, the labelled molecules were no longer released by the enzymes. Hence, during incubation at 37 °C, release of the ^{125}I-labelled transferrin became resistant to the action of the enzymes, most probably because during incubation at this temperature the molecules are actually taken into the cell. If this is true, then, the majority of the transferrin molecules within the cell are not free in the cytoplasm but presumably are bound to intracellular membranes or are within endocytotic vesicles. Thus, although prolonged incubation of reticulocytes with MalNEt and trypsin led to haemolysis with release of 80 % of the ^{59}Fe and haemoglobin of the cells, relatively little transferrin, which has a molecular weight only slightly greater than haemoglobin [13], was released (Fig. 5) indicating that it was in some way bound within the cell. Morphological evidence for transferrin uptake by endocytosis has been obtained previously using transferrin coupled to ferritin [8]. The intracellular distribution of transferrin in both intact and haemolysed cells was confirmed in the present work by autoradiography (Fig. 6).

If the mechanism of transferrin uptake by reticulocytes is by endocytosis then it is likely that transferrin release results from the reverse process, exocytosis. Proteolytic digestion of the cells did not inhibit the process of transferrin release but instead led to the total release of about 10 % more transferrin than in control cells (Fig. 4). This may be due to proteolytic release of transferrin receptors from the cells so that when equilibrium is reached there is more transferrin in the medium and less on the cells because of the deficit in transferrin-binding sites on the cells.

The results from the study on enzyme-treated whole cells and sealed ghosts (Fig. 3) support the hypothesis that transferrin uptake and release occurs by endocytosis and exocytosis. The reduction in transferrin binding to receptors, as measured by the amount of ^{125}I-labelled transferrin eluted close to the void volume during gel filtration was much greater when intact cells were incubated with trypsin than when sealed ghosts were used. In the case of intact cells, a continuing process of endocytosis and exocytosis would have allowed a large proportion of the transferrin receptors to be digested by an enzyme acting on the outer surface of the cell membrane. However, if endocytosis and exocytosis ceased after haemolysis of the cells during production of the ghosts then the receptors within sealed ghosts would be unavailable to the enzyme. Hence, when the membrane was subsequently solubilized, relatively more intact

transferrin-binding sites would be present in the extract of ghosts than in that of intact cells. The release of ^{125}I-labelled transferrin from reticulocyte ghosts is much slower than from intact cells [17] probably because exocytosis is inhibited by the production of the ghosts.

The degree of initial reflux of ^{59}Fe from the cells which had taken up ^{125}I/^{59}Fe-labelled transferrin at 4 °C was similar to that of ^{125}I, indicating that the ^{59}Fe initially released was transferrin bound. As reincubation continued, the amount of ^{59}Fe in the medium became progressively less due to uptake by the cells. After 30 min reincubation less than 5 % of the ^{59}Fe remained in the medium. This shows that reticulocytes can remove iron from both of the iron-binding sites of transferrin and casts doubt on the theory that reticulocytes take up iron preferentially from one site as proposed by Fletcher and Huehns [26].

The observation that incubation of reticulocytes with neuraminidase did not affect transferrin or iron uptake or release indicates that cell membrane sialic acid residues are not involved in these processes and that if sialic acid is present on the transferrin receptor it is of no importance in its interaction with transferrin. Furthermore, since sialic acid has been shown to be the major contributor to the surface charge of red cells [27] it is highly unlikely that the effects of proteolytic enzymes on transferrin and iron uptake are due to changes in membrane surface charge.

The present results obtained with neuraminidase disagree with those of Jandl *et al.* [2] who found that preincubation of human reticulocytes with receptor-destroying enzyme produced an inhibition of iron uptake. The reason for this difference is not certain. The proportion of sialic acid released from the cells was not reported by Jandl *et al.* Hence, it is not possible to compare the degree of change to the cell membrane produced in the two experiments. Also, it is possible that the earlier preparation of neuraminidase possessed a significant amount of proteolytic activity.

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