Biochimica et Biophysica Acta, 642 (1981) 119—134

BBA 79162

INHIBITION OF RETICULOCYTE IRON UPTAKE BY NH_4Cl AND CH_3NH_2

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(Received October 20th, 1980)

Key words: Transferrin; Iron uptake; NH_4Cl; Methylamine; (Reticulocyte)

Summary

The aim of this investigation was to test the hypothesis that elevation of intracellular pH would inhibit iron uptake by reticulocytes. The experiments were performed with rabbit reticulocytes and iron bound to rabbit transferrin. Incubation of the cells with NH_4Cl, $(NH_4)_2CO_3$, CH_3NH_2 and $(CH_3)_2NH$ was used in an attempt to increase intracellular pH. These substances were all found to inhibit iron uptake by reticulocytes. The mechanism of action of NH_4Cl and CH_3NH_2 was investigated in detail. Similar results were found with both reagents. They inhibited iron uptake in a concentration-dependent manner, but produced a small increase in the cellular uptake of transferrin. The onset of action was rapid and the effect was reversible. There was no decrease in the number of transferrin-binding sites per cell and their apparent affinity for transferrin increased slightly, while the efficiency of iron removal from transferrin per binding site diminished greatly. The rate of transferrin release from reticulocytes was unaffected. NH_4Cl did not affect the rate of iron release from transferrin in a cell-free system. Incubation of reticulocytes with 10 mM NH_4Cl or CH_3NH_2 was found to produce an increase in intracellular pH of 0.05—0.15 pH units. The intracellular pH determined by use of the weak acid 5,5-dimethyloxazolidine-2,4-dione was significantly higher than that obtained with the weak base $(CH_3)_2NH$. By transmission electron microscopy it was shown that reticulocytes treated with NH_4Cl or CH_3NH_2 have enlarged intracellular vesicles. The results are considered to support the hypothesis that iron release from transferrin in reticulocytes occurs as a result of protonation of the transferrin within intracellular vesicles. According to this hypothesis, weak bases such as NH_3 and CH_3NH_2 inhibit iron release by neutralizing H^+ within the vesicles.

Introduction

The uptake of transferrin-bound iron by immature erythroid cells involves an interaction between the protein and cell membrane receptors followed by release of the iron from transferrin and its incorporation into haemoglobin [1—3]. The mechanism by which the iron is released from its transport protein is unknown. However, several hypotheses have been proposed. These include a conformational change in the protein, reduction of iron, chelation of iron, release of HCO_3^- or protonation of the iron-transferrin complex [4]. Experimental evidence favours the last two of the possibilities. Several workers have shown that HCO_3^- (or CO_3^{2-}) is released from transferrin in parallel with iron uptake by reticulocytes and it has been proposed that the release of HCO_3^- which allows the release of iron is catalysed enzymatically [5—7]. However, since HCO_3^- is always released from transferrin when the iron is released, it is not possible to say whether HCO_3^- release is the primary event. The other hypothesis, that iron release results from protonation of the iron-transferrin complex, is supported by the observations that iron binding by transferrin is dependent on pH [8,9] and that the rate of iron release from transferrin which is mediated by phosphate compounds and certain iron chelators is proportional to the H^+ concentration of the solution [10—12].

There is no evidence that protonation of transferrin is the mechanism responsible for iron release from the protein during iron uptake by erythroid precursors or other types of cell. However, if it is, elevation of intracellular pH should lead to a reduction in the rate of iron uptake. The present experiments were undertaken to test this prediction and, hence, to shed more light on the mechanism by which iron is released from transferrin during uptake by immature erythroid cells. Iron and transferrin uptake by rabbit reticulocytes was measured in the presence and absence of NH_4Cl and CH_3NH_2 which can cause an increase of intracellular pH [13]. NH_3 formed from NH_4^+, CH_3NH_2 and other weak bases diffuses through the cell membrane and then reacts with intracellular H^+, leading to an elevation of the pH.

Materials and Methods

Radioisotopes. ^{59}Fe ($^{59}FeCl_3$, 10—30 μCi/μg), ^{125}I ($Na^{125}I$, carrier free) and [^{14}C]DMO (5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione, 435 μCi/mg) were purchased from the Radiochemical Centre, Amersham, U.K. $(^{14}CH_3)_2NH \cdot HCl$ (47 mCi/mmol) was obtained from New England Nuclear, Boston, MA.

Reticulocytes. Reticulocytosis was induced in adult rabbits by treatment with phenylhydrazine [14]. Blood was collected from the marginal ear vein using heparin as an anticoagulant between 3 and 7 days after the last injection of phenylhydrazine. The cells were washed three times with 0.15 M NaCl and were then suspended in phosphate-buffered saline (1 vol. of 310 mosM sodium phosphate, pH 7.4—7.5, and 10 vol. of 0.15 M NaCl).

Isolation and labellings of transferrin. Transferrin was isolated from rabbit plasma by ion-exchange chromatography and gel filtration and was labelled with ^{125}I and ^{59}Fe as described earlier [14].

Incubation procedures. Transferrin and iron uptake by reticulocytes was mea-

sured by incubating the cells suspended in phosphate-buffered saline at a haematocrit of 15—20% with ^{59}Fe- ^{125}I-labelled transferrin at 37°C, followed by washing three times in ice-cold 0.15 M NaCl, transfer of the cells to fresh counting tubes, haemolysis with water and counting radioactivity in a three-channel gamma-scintillation counter. The release of transferrin from reticulocytes was measured by incubating the cells with ^{125}I-labelled transferrin for 20 min at 37°C followed by washing the cells four times in ice-cold 0.15 M NaCl. The cells were then resuspended in ice-cold phosphate-buffered saline containing unlabelled transferrin (1 mg/ml) and reincubated at 37°C. After varying lengths of time, samples of the cell suspension were transferred to tubes containing ice-cold 0.15 M NaCl, mixed, centrifuged and the radioactivity counted in cell and supernatant layers in order to measure the amount of ^{125}I-labelled transferrin released from the cells. The effects of NH_4Cl and CH_3NH_2 on iron and transferrin uptake and transferrin release were determined by adding varying amounts of 0.15 M solutions of the reagents to the cell suspensions prior to incubation at 37°C. The pH of the NH_4Cl and CH_3NH_2 solutions was adjusted to 7.4 by the addition of 0.15 M NaOH prior to use in the experiments.

Electron microscopy. Previously described methods were used for the preparation and examination of reticulocytes by transmission electron microscopy [15].

Analytical methods. Reticulocytes were counted on dry smears by staining with new methylene blue. Packed cell volume was determined by the microhaematocrit method. Intracellular pH was measured by the use of [^{14}C]DMO and ($^{14}CH_3$)NH as described by Warth and Desforges [16]. After incubation with these labelled substances for 20 min at 37°C, cells and incubation solution were separated by centrifugation, the pH of the solution measured with a glass electrode and radioactivity counted in the solution and cellular fraction. The extracellular fluid volume of the cellular fraction was determined by measurement of packed cell volume and the intracellular fluid volume by utilizing the value 0.59 as the fraction of solvent water in the cells [16]. Standard methods were used to measure ATP [17] and methaemoglobin [18].

Results

Transferrin and iron uptake by reticulocytes

When reticulocytes are incubated at 37°C with ^{59}Fe- and ^{125}I-labelled transferrin, iron is taken up by the cells in a linear manner while the uptake of the labelled transferrin, although initially fast, soon slows to reach a plateau level after 10—15 min (Fig. 1). Addition of 15 mM NH_4Cl or CH_3NH_2 to the incubation medium markedly inhibited iron uptake but produced little change in transferrin uptake (Fig. 1). This effect was concentration dependent. Fig. 2 illustrates the results obtained when the concentration of NH_4Cl was varied from 2 to 20 mM. Similar results were obtained with varying concentrations of CH_3NH_2. As the concentrations were raised there was an increasing degree of inhibition of iron uptake. In contrast, transferrin uptake was slightly enhanced at NH_4Cl or CH_3NH_2 concentrations of 2—10 mM and only at 20 mM was there evidence of any decrease in the amount of transferrin taken up by the cells. In another experiment, it was shown that $(NH_4)_2CO_3$ $(NH_4)_2SO_4$ and also $(CH_3)_2$-

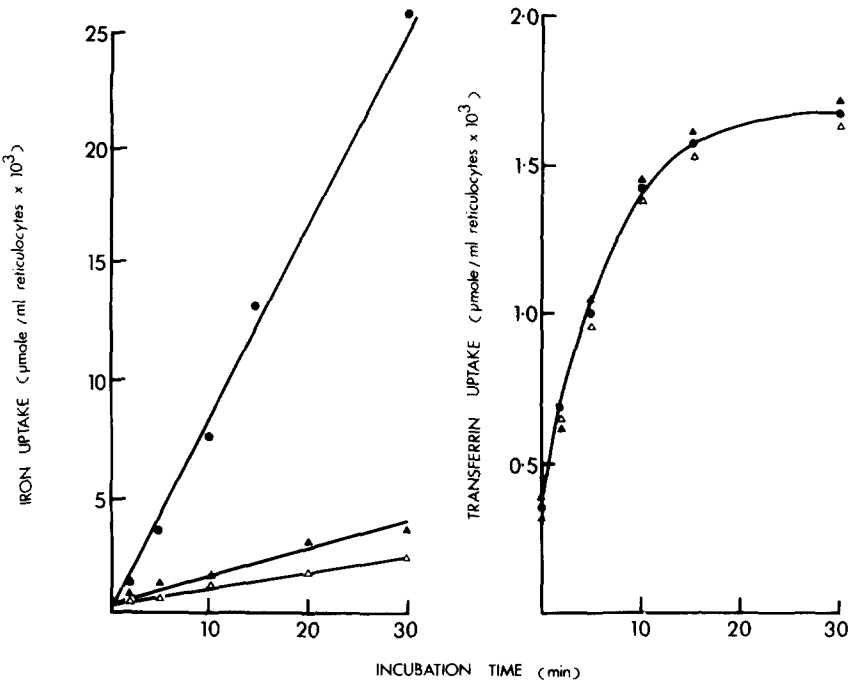


Fig. 1. Effect of NH_4Cl (▲) and CH_3NH_2 (△) on iron and transferrin uptake by rabbit reticulocytes. The reagents were used at a concentration of 16 mM. The reticulocyte count was 51%. The incubation was performed as described in the text using ^{125}I- and ^{59}Fe-labelled transferrin at a concentration of 0.8 mg/ml. Control incubation: ●——●.

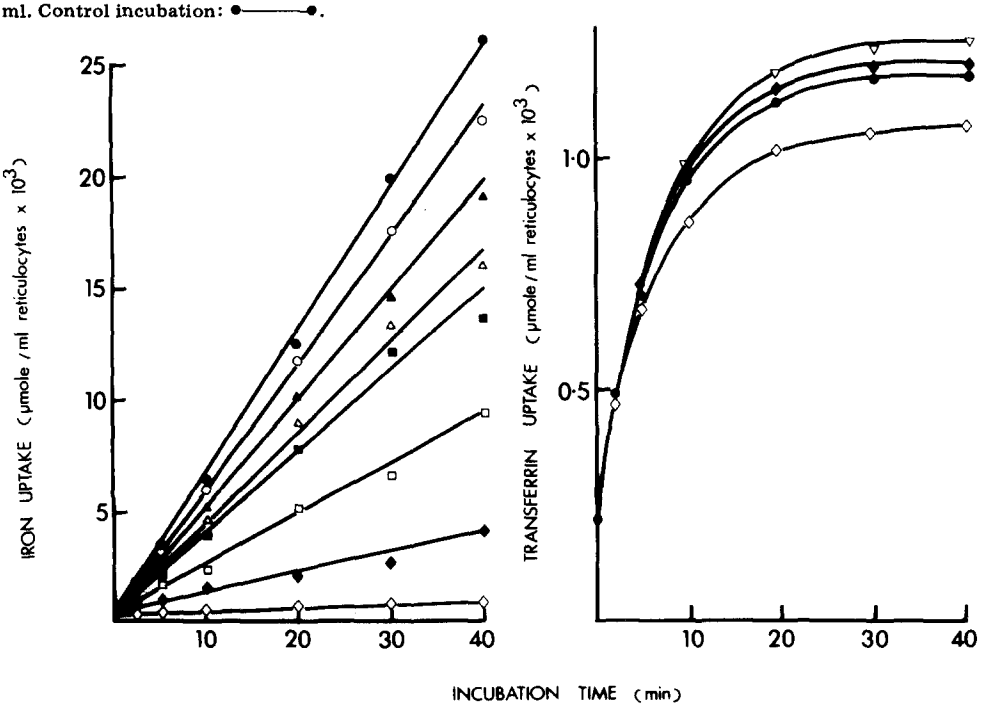


Fig. 2. Effects of varying concentrations of NH_4Cl on iron and transferrin uptake by reticulocytes. NH_4Cl was added to the cell suspension (30% reticulocytes) prior to incubation with labelled transferrin (1.3 mg/ml). The NH_4Cl concentrations were: 0 (●), 2 mM (○), 4 mM (▲), 6 mM (△), 8 mM (■), 10 mM (□), 15 mM (◆) and 20 mM (◇). No apparent difference in effect on transferrin uptake was observed with NH_4Cl concentrations of 2—20 mM. Hence, these results are indicated by a single curve (▽).

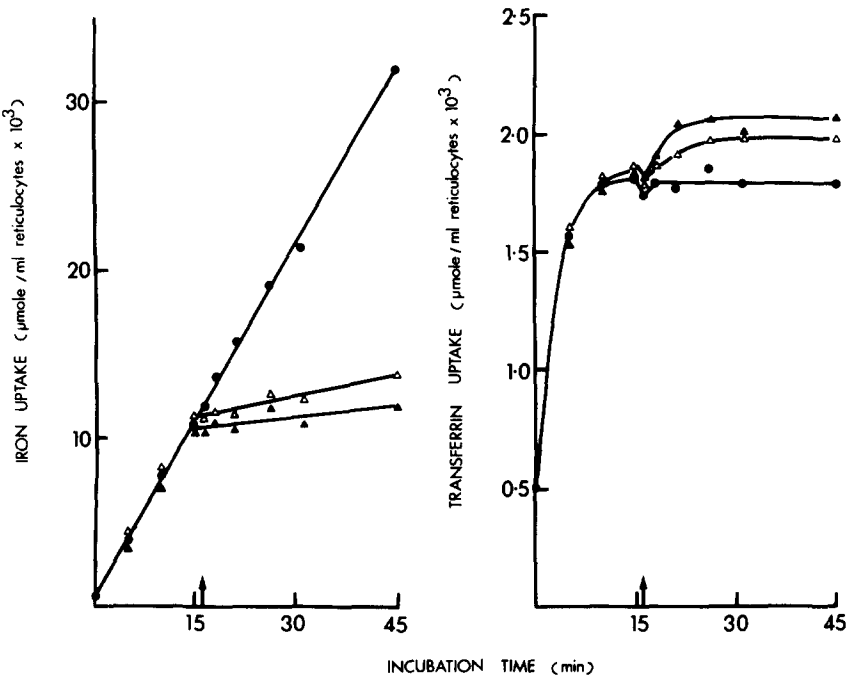


Fig. 3. Rate of onset of effects of NH_4Cl (▲) and CH_3NH_2 (△) on iron and transferrin uptake by rabbit reticulocytes. The reagents were added at the time indicated by the arrow to give a concentration of 16 mM. Isotonic NaCl was added to the control incubation mixture (●). The reticulocyte count was 25% and the transferrin concentration was 0.5 mg/ml.

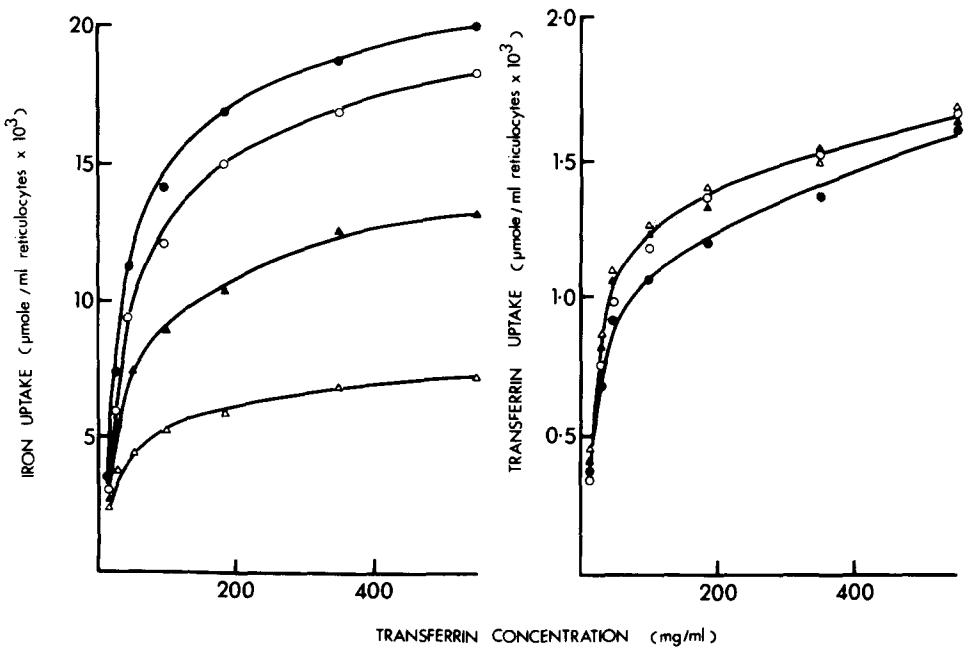


Fig. 4. Effect of NH_4Cl on iron and transferrin uptake by reticulocytes incubated with the indicated concentrations of diferric transferrin. The cells (46% reticulocytes) were incubated with the labelled transferrin at 37°C for 30 min. Each point is the mean of duplicate incubations. The NH_4Cl concentrations were: 0 (●), 3 mM (○), 8 mM (▲) and 16 mM (△).

NH had a similar effects to those observed with NH_4Cl and CH_3NH_2.

The rate of onset of the effects of NH_4Cl and CH_3NH_2 was investigated by adding the reagents during the period of incubation of reticulocytes with ^{125}I- and ^{59}Fe-labelled transferrin. Inhibition of iron uptake was apparent within 30 s and continued during subsequent incubation (Fig. 3). In contrast, the amount of labelled transferrin bound by the cells increased at the same time as iron uptake was inhibited and rose to a level significantly above that of control cells incubated in the absence of the reagents (Fig. 3).

The mechanism of action of NH_4Cl and CH_3NH_2 was further investigated by incubating reticulocytes with varying concentrations of labelled transferrin in the absence or presence of the inhibitors at concentrations of 3, 8 and 16 mM. Fig. 4 shows the results obtained with NH_4Cl. The results with CH_3NH_2 were similar. There was a concentration-dependent inhibition of iron uptake, but some enhancement of transferrin uptake which was also concentration dependent at the lower concentrations of transferrin but independent of concentration of NH_4Cl or CH_3NH_2 when the transferrin concentration was above 100 μg/ml. The data from this experiment were analysed by the method of Scatchard as described earlier [19] to give estimates of the number of transferrin-binding sites per reticulocyte and the association constant of these sites for transferrin. The maximum rate of iron uptake by the cells was also calculated by the same method. As shown in Table I, neither NH_4Cl or CH_3NH_2 had any effect on the number of transferrin-binding sites per cell, which averaged 85 000 sites per cell, assuming that the sites are univalent for transferrin. In contrast, the apparent association constants of the sites for transferrin were affected by the reagents, increasing as their concentration was raised from a

TABLE I

EFFECT OF NH_4Cl AND CH_3NH_2 ON THE MAXIMUM TRANSFERRIN UPTAKE (B_{max}) AND APPARENT ASSOCIATION CONSTANT (K_A) AND MAXIMUM RATE OF IRON UPTAKE (B_{max}) BY RABBIT RETICULOCYTES

The results were calculated as described in the text from the data of the experiment illustrated in part in Fig. 4. Also given is the ratio of the maximum rate of iron uptake to the maximum transferrin uptake by the cells (B_{max}(Fe) : B_{max}(TN)).

	Transferrin uptake		Iron uptake	B_{max}(Fe) : B_{max}(TN)
	B_{max} (molecules/cell) ($\times 10^{-5}$)	K_A (M^{-1})($\times 10^7$)	B_{max} (molecules/cell per h) ($\times 10^{-5}$)	
Control				
1	0.81	0.40	22.0	27
2	0.84	0.36	22.4	27
NH_4Cl				
3 mM	0.81	0.39	22.0	27
8 mM	0.88	0.49	12.6	14
16 mM	0.87	0.51	6.6	8
CH_3NH_2				
3 mM	0.83	0.39	21.8	27
8 mM	0.88	0.47	10.0	11
16 mM	0.88	0.48	5.4	6

mean of $0.38 \cdot 10^7$ l/mol for the controls to about $0.5 \cdot 10^7$ l/mol when the reagents were present at 16 mM. The maximal rate of iron uptake was inhibited in a concentration-dependent manner from $22 \cdot 10^5$ molecules/cell per h in the control cells to about $6 \cdot 10^5$ molecules/cells per h when the inhibitors were present at 16 mM. On the basis of these data and the fact that each transferrin molecule binds two atoms of iron, it was calculated that, on average, each transferrin-binding site was associated with the release of all the iron from about 13 transferrin molecules per h in control cells but only three to four transferrin molecules in the cells incubated in the presence of the highest concentrations of inhibitors, i.e., the efficiency of function of the receptors appeared to be markedly impaired without any reduction in their numbers and in the presence of some increase in their apparent affinity for transferrin.

The reversibility of the effects of NH_4Cl and CH_3NH_2 was investigated by incubating reticulocytes for 15 min at 37°C in the presence of NaCl (control) or 15 mM concentrations of the reagents. Subsequently, the cells were washed twice by incubation for 10 min at 37°C in 20 vol. phosphate-buffered saline. They were then suspended in the same solution and incubated with labelled transferrin. Other samples of cells were not washed until after the initial incubation with the reagents. The effects of NH_4Cl and CH_3NH_2 on iron and transferrin uptake were found to be completely reversed by this procedure. Fig. 5 shows the results with 10 mM NH_4Cl. Similar results were obtained with CH_3NH_2.

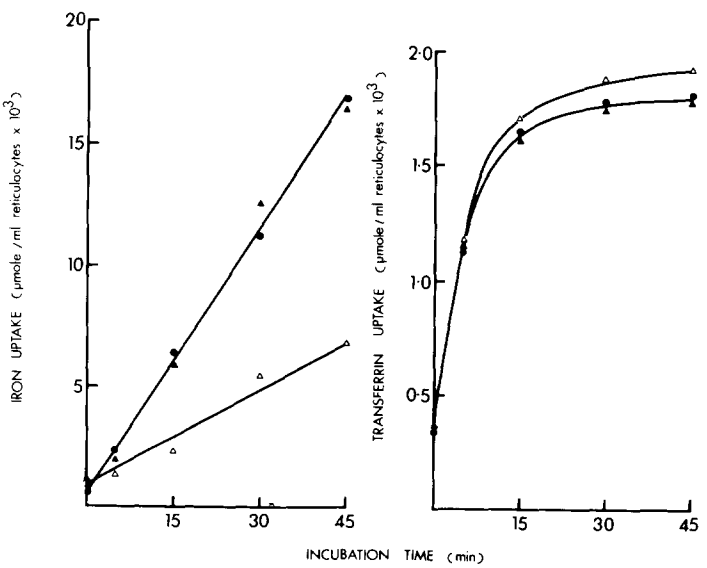


Fig. 5. Reversal of the effect of NH_4Cl on iron and transferrin uptake by reticulocytes. Reticulocytes (55%) were incubated with 0.15 M NaCl (●) or 15 mM NH_4Cl in NaCl (▲) for 15 min, washed twice in phosphate-buffered NaCl (see text) and then incubated with labelled transferrin (1.5 mg/ml). Another sample of cells was incubated with 15 mM NH_4Cl (△) but was not washed before incubation with labelled transferrin.

Transferrin release from reticulocytes

The effects of NH_4Cl and CH_3NH_2 on the release of transferrin from reticulocytes were investigated by incubating the cells in the presence or absence of the reagents, washing with ice-cold NaCl and reincubating again in the presence or absence of the reagents. When reincubation was performed in NaCl, the rate and amount of transferrin release from the cells were the same whether or not the reagents had been present during the uptake of the labelled transferrin (results not shown). Similarly, the presence of either reagent in the reincubation solution had little effect on the rate of transferrin release from the cells, but decreased the total amount of labelled transferrin released after 15—30 min incubation by about 10% (Fig. 6A). In another experiment, the effect of trypsin on transferrin release from cells which had taken the protein up in the presence of 15 mM NH_4Cl was investigated using *N*-ethylmaleimide to block the normal release process, as described earlier [20]. Transferrin release was markedly inhibited by *N*-ethylmaleimide, to a slightly greater extent in the NH_4Cl-treated cells than in the controls (Fig. 6B). Trypsin produced a small increase in transferrin release, but the effect was similar in test and control cells and afer 30 min only about 30% of the transferrin had been released, compared with about 90% from those cells not exposed to *N*-ethylmaleimide. These

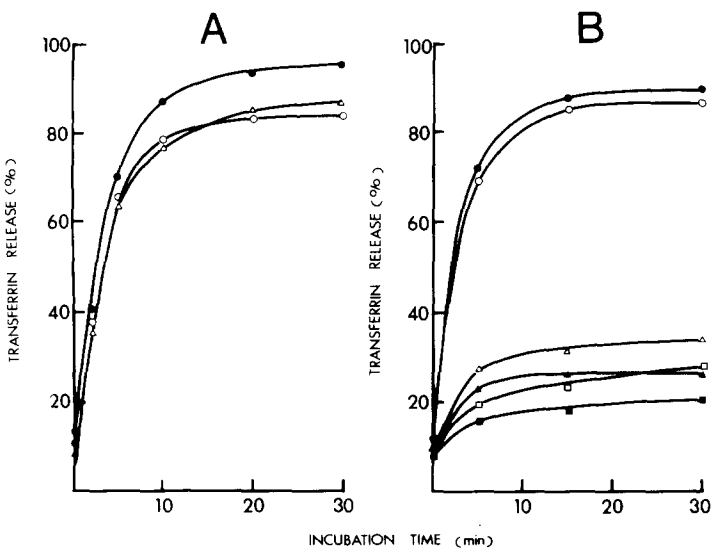


Fig. 6. Effect of NH_4Cl and CH_3NH_2 on transferrin release from reticulocytes. Results obtained when reticulocytes prelabelled with ^{125}I-labelled transferrin were incubated in the presence of 15 mM NH_4Cl (○) or 15 mM CH_3NH_2 (△) as compared with a control cell suspension (●) where neither of these reagents was present. (B) Effects of trypsin (0.5 mg/ml) and *N*-ethylmaleimide (5 mM) on ^{125}I-labelled transferrin release from reticulocytes which had taken up the labelled transferrin either in the presence or absence of NH_4Cl (15 mM). After incubation with the labelled transferrin, the cells were washed with ice-cold 0.15 M NaCl and then reincubated in the presence or absence of trypsin and *N*-ethylmaleimide. Trypsin had no effect on transferrin release when *N*-ethylmaleimide was not present. (○) Uptake, no addition; release, with or without trypsin (no *N*-ethylmaleimide). (●) Uptake, NH_4Cl: release, with or without trypsin (no *N*-ethylmaleimide). (△) Uptake, no addition: release, *N*-ethylmaleimide and trypsin. (▲) Uptake, no addition: release, *N*-ethylmaleimide only. (□) Uptake, NH_4Cl: release, *N*-ethylmaleimide and trypsin. (■) Uptake, NH_4Cl: release, *N*-ethylmaleimide only.

results showed that the release of transferrin taken up by reticulocytes in the presence of NH_4Cl was inhibited by *N*-ethylmaleimide and that as in the control cells the transferrin was not available for release by the action of trypsin.

Iron release from transferrin

The major effect of NH_4Cl and CH_3NH_2 which was observed in the above experiments was inhibition of iron but not transferrin uptake by reticulocytes. Presumably, this is due to inhibition of iron release from transferrin molecules after their uptake by the cells. It was therefore necessary to determine whether the reagents had a direct inhibitory effect on iron release from the protein. This was investigated by incubating ^{59}Fe- and ^{125}I-labelled transferrin in a dialysis sac with a freshly prepared haemolysate of reticulocytes plus either dipyridyl (2 mM) or desferrioxamine (1 mM) as an iron acceptor, and either 15 mM NH_4Cl or an equivalent amount of NaCl. The sac was incubated at 37°C in a flask containing 2 mM dipyridyl or 1 mM desferrioxamine in phosphate-buffered saline (pH 6.8). 15 mM NH_4Cl was also present in the dialysate in the appropriate samples. The haemolysate was prepared by haemolysing 1 ml packed cells (30% reticulocytes) with 4 ml of 15 mosM phosphate buffer, pH 7.4, followed by centrifugation at 40 000 × *g* for 1 h and adjustment of the pH of the supernatant solution to pH 6.8 with 0.1 M HCl. For each incubation, 1 mg labelled transferrin was incubated with 0.16 ml of this solution. Samples of the dialysate were removed and counted for radioactivity after varying periods of incubation. The rate of iron release from transferrin into the dialys-

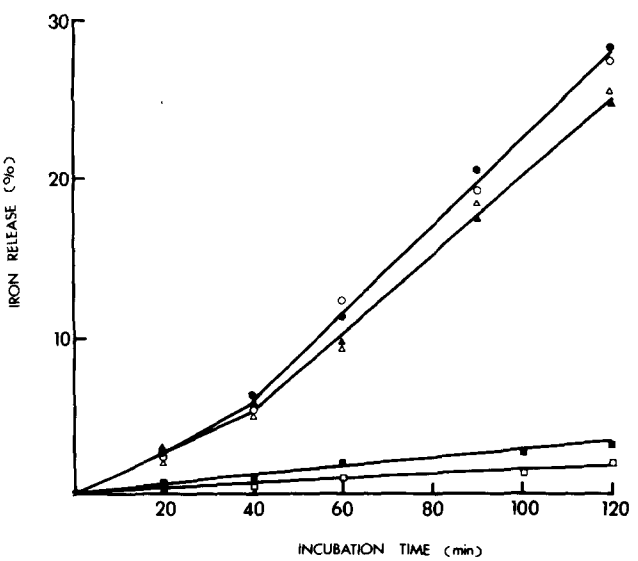


Fig. 7. Effect of NH_4Cl (15 mM) on iron release from rabbit transferrin in a cell-free system. Iron release was mediated by a haemolysate from rabbit reticulocytes (see text for details). The figure shows the percentage of the iron present on the transferrin which was released during incubation at 37°C and at pH 6.8 in the presence (△,●) or the absence (▲,●) of NH_4Cl using desferrioxamine (▲,△) or α,α-dipyridyl (●,○) as the iron acceptor. Also shown is the release which occurred when the haemolysate was replaced by phosphate-buffered saline using desferrioxamine (□) or α,α-dipyridyl (■) as the iron acceptor. No NH_4Cl was present in these two incubations.

ate was the same in the presence of NH_4Cl as in its absence, and was very similar whether dipyridyl or desferrioxamine was used as the iron acceptor (Fig. 7). Iron release from transferrin appeared to be dependent on the presence of the haemolysate, since little release occurred in its absence even though dipyridyl or desferrioxamine was present.

Cellular pH, ATP and methaemoglobin levels

Intracellular pH was measured in two experiments using control cells incubated for 20 min in phosphate-buffered saline and comparable samples of cells incubated in phosphate-buffered saline containing 10 mM NH_4Cl or 10 mM CH_3NH_2. In the first experiment, the weak acid [^{14}C]DMO was used. Incubation with NH_4Cl or CH_3NH_2 was found to raise the intracellular pH by an average of 0.05 pH units (Table II). The question was then posed as to whether the cell interior of reticulocytes is homogeneous with regard to pH. In order to investigate this, measurements of intracellular pH were made using the weak base, $(^{14}CH_3)_2NH$, as well as by using [^{14}C]DMO. Several workers had demonstrated that both methods will give the same result if the cell interior is homogeneous, but if it is heterogenous the acid will give a value closer to the highest pH within the cell system and the base a value closer to the lowest pH [21—24]. The results of the present investigation are summarized in Table III. The values obtained with $(^{14}CH_3)_2NH$ were consistently lower than those with [^{14}C]DMO, the mean difference being 0.39 pH units. Incubation with NH_4Cl or CH_3NH_2 was again found to raise the intracellular pH determined with [^{14}C]-DMO by about 0.05 pH units. A greater rise, 0.15—0.17 pH units, was observed when the intracellular pH was measured by the use of $(^{14}CH_3)_2NH$.

Incubation of reticulocytes with 10 mM NH_4Cl or 10 mM CH_3NH_2 produced no change in the methaemoglobin content of the cells and less than 10% reduction in ATP when compared with the values for samples of cells incubated in the absence of these reagents (Table IV).

Electron microscopy

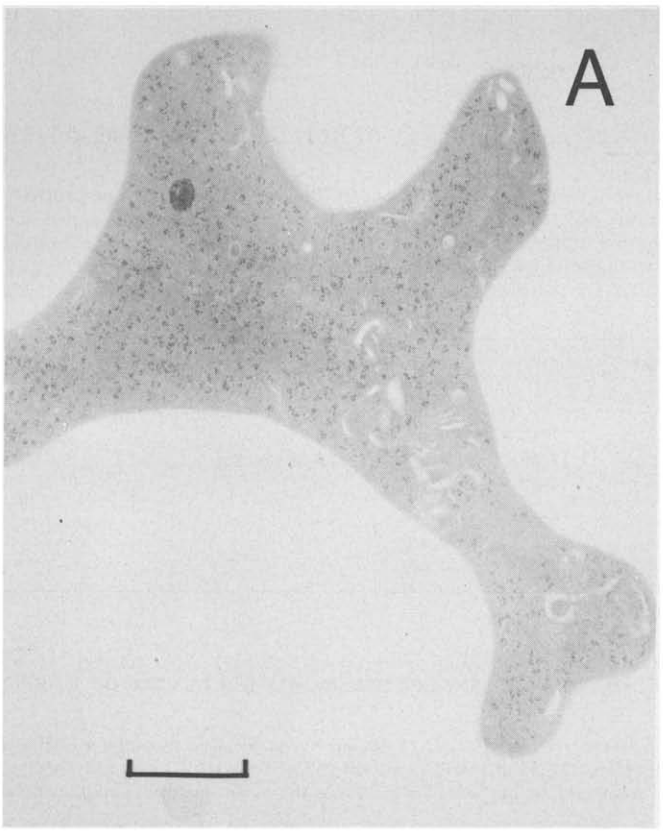
Thin sections of cells which had been incubated with 5 and 15 mM NH_4Cl or CH_3NH_2 for 20 min at 37°C and control cells incubated without these reagents were examined by electron microscopy. Many of the cells incubated with the

TABLE II

EFFECT OF NH_4Cl AND CH_3NH_2 ON INTRACELLULAR pH OF RETICULOCYTES AS MEASURED WITH [^{14}C]DMO

The results are the means ± S.E. of four determinations made on different samples of cells. The cell samples (43—57% reticulocytes) were incubated for 10 min at 37°C with 10 mM NH_4Cl or CH_3NH_2 in phosphate-buffered saline or with the buffer alone, then [^{14}C]DMO was added and the incubation continued for a further 20 min. Intracellular pH (pH_i) and extracellular pH (pH_e) were determined as described in the text.

Treatment of cells	pH_i	pH_e	ΔpH ($pH_i - pH_e$)
Control	7.44 ± 0.018	7.47 ± 0.015	−0.03 ± 0.010
NH_4Cl	7.50 ± 0.014	7.45 ± 0.026	+0.05 ± 0.021
CH_3NH_2	7.48 ± 0.021	7.46 ± 0.032	+0.02 ± 0.013



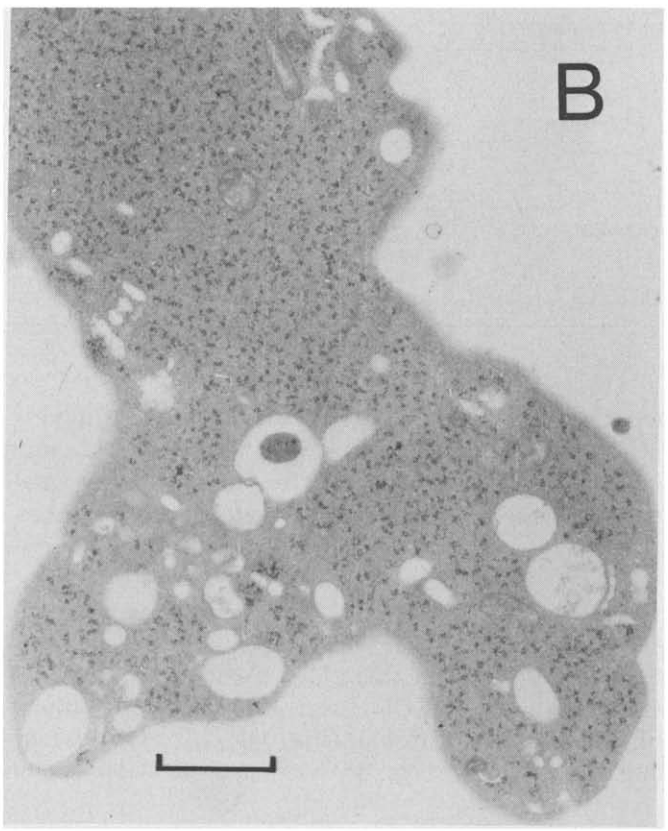


Fig. 8. Electron micrographs of a control reticulocyte (A) and a reticulocyte which had been incubated at 37°C for 20 min in the presence of 15 mM NH_4Cl (B). Bar represents 1 μm.

TABLE III

EFFECT OF NH_4Cl AND CH_3NH_2 ON INTRACELLULAR pH OF RETICULOCYTES AS MEASURED WITH [^{14}C]DMO OR $(^{14}CH_3)_2NH$

The cell samples (42% reticulocytes) were incubated for 10 min at 37°C with 10 mM NH_4Cl or CH_3NH_2 in phosphate-buffered saline or with the buffer alone, then [^{14}C]DMO or $(^{14}CH_3)_2NH$ was added and the incubation was continued for another 20 min. Extracellular pH (pH_e) and intracellular pH (pH_i) were determined as described in the text. The rate of iron uptake was measured in other samples of cells treated in the same way as those used for the pH measurements. Each value is the mean of duplicate determinations.

pH_i Method	Treatment of cells	pH_i	pH_e	Rate of iron uptake (μmol/ml reticulocytes per h)
[^{14}C]DMO	Control	7.44	7.49	29
	NH_4Cl	7.49	7.49	6.3
	CH_3NH_2	7.50	7.49	5.5
$(^{14}CH_3)_2NH$	Control	6.98	7.50	27
	NH_4Cl	7.13	7.49	6.1
	CH_3NH_2	7.15	7.50	5.5

TABLE IV

EFFECT OF NH_4Cl AND CH_3NH_2 ON THE ATP AND METHAEMOGLOBIN LEVELS OF RABBIT RETICULOCYTES

The cell samples (56% reticulocytes) were incubated for 0, 15 or 30 min at 37°C in phosphate-buffered saline with or without the addition of 15 mM NH_4Cl or 15 mM CH_3NH_2. Cellular ATP and methaemoglobin levels were then measured as described in the text. Each value is the mean of duplicate determinations.

Treatment of cells	Incubation time (min)		
	0	15	30
ATP level (μmol/ml cells)			
Control	3.2	3.2	3.0
NH_4Cl	3.2	3.0	2.8
CH_3NH_2	3.0	2.8	2.9
Methaemoglobin level (% total haemoglobin)			
Control	1.9	2.2	2.0
NH_4Cl	1.9	1.7	1.4
CH_3NH_2	2.2	1.5	1.7

reagents contained large vacuoles (Fig. 8). The large vacuoles were confined to reticulocytes, as judged by the presence of ribosomes and mitochondria, none being seen in mature erythrocytes. Control reticulocytes showed many small vesicles but no vacuoles of the type seen in the treated cells. Mature erythrocytes contained no vesicles.

Discussion

The results of the experiments described in this paper show that NH_4Cl and CH_3NH_2 (and also other ammonium salts and $(CH_3)_2NH$) inhibit iron uptake by reticulocytes without inhibiting transferrin uptake (Fig. 1). This effect occurred immediately after adding the reagents (Fig. 3), was concentration depen-

dent (Fig. 2) and was completely reversible (Fig. 5). The results suggest that the inhibitory action was exerted on the process by which iron is released from the transferrin molecule. However, there was no evidence of a direct inhibition of iron release mediated by reticulocyte cytosol (Fig. 7).

The rates of transferrin uptake and release, as indicated by the initial slopes of the uptake and release phases of transferrin-reticulocyte interaction (Figs. 1, 2 and 6) were not affected by the reagents. However, the maximum or plateau level of transferrin taken up by the cells was increased while the maximum amount of transferrin lost during the release process was diminished. Hence, in the steady-state situation, more transferrin was bound by the cells in the presence of NH_4Cl, CH_3NH_2 and related compounds, than in their absence. In other words, these reagents appeared to increase the affinity of the cells for transferrin, probably by increasing the affinity of transferrin receptors for the protein. This conclusion is confirmed by the measurements of receptor numbers and affinity (Table I), which showed no increase in number but an increase in the apparent association constant of the receptors for transferrin. The results of this experiment also indicated that the rate of cellular uptake of iron per receptor diminished as the NH_4Cl and CH_3NH_2 concentrations were raised (Table I). The increase in apparent affinity of receptors for transferrin and the decreased rate of iron release from the protein are probably related phenomena. The affinity of receptors for diferric transferrin is greater than that for apotransferrin [25]. It is likely that the action of the agents used in the present experiments was to inhibit iron release from transferrin which had bound normally to receptors and had been taken up normally by the cells, and that the relatively greater amount of iron-transferrin present in the treated cells than in the controls resulted in the increase in the measured association constant of the receptors for transferrin.

Earlier work has shown that transferrin is taken into reticulocytes by endocytosis [26—28]. In terms of uptake of labelled transferrin this process is represented by the increase in cell-bound ^{125}I-labelled transferrin which occurs during the first 10—15 min of incubation at 37°C (Figs. 1 and 2). It is inhibited at lower incubation temperatures and is almost absent at 4°C [28]. Since the initial rate of uptake of transferrin during incubation at 37°C was not affected by NH_4Cl or CH_3NH_2, it is probably that these reagents had no effect on the endocytosis of the protein. In addition, transferrin taken up by reticulocytes in the presence of NH_4Cl was not released from the cells by the action of trypsin (Fig. 6B). It is likely that this occurred because the protein was protected from the action of the enzyme by being contained within the cells in endocytotic vesicles.

Although no evidence for inhibition of endocytosis by NH_3 was found in the present work, it should be noted that NH_3 has been reported to inhibit the clustering of α-2-macroglobulin and epidermal growth factor on the surface of fibroblasts [29] and the uptake of diphtheria toxin [30,31] and certain viruses [32] by other types of cell. The mechanism of inhibition in these situations is uncertain. However, there is evidence that inhibition of virus entry into cells may be due to an increase in intralysosomal pH which causes inhibition of a pH-dependent membrane fusion reaction [33].

Several investigators have shown that NH_3, CH_3NH_2 and other weak bases are

taken up by a variety of cells and concentrated within lysosomes, leading to an elevation of intralysosomal pH [34—36]. The bases accumulate in the lysosomes due to ion trapping by reacting with H^+ which is present in lysosomes in high concentrations. The resulting ionized forms of the bases are unable to penetrate the lysosomal membrane. The accumulation leads to osmotic swelling of the lysosomes [37,38]. It is likely that the large intracellular vesicles observed in the present investigation are the result of accumulation of the bases within intracellular vesicles. This would be accompanied by an increase in the membrane surface area of the individual vesicles as a result of fusion of preexisting vesicles or by the addition of membrane from other sources such as the external surface of the cell. As with lysosomes, accumulation of the bases in the vesicles is probably due to ion trapping, suggestion that there is a ready availability of H^+ inside the vesicles. This must be due to the passage of H^+ through the vesicular membrane. Such ions would presumably be derived from cellular metabolism. Under normal circumstances, H^+ within endocytotic vesicles may react with transferrin leading to iron release, as has been shown to occur with cell-free solutions of transferrin [10—12]. Weak bases would inhibit this process by combining with H^+. All of the results found in the present work are compatible with this hypothesis, including the rapid onset of the action of the bases, since it has been shown that they can raise the intralysosomal pH of living macrophages extremely rapidly [36].

The results obtained for the measurement of intracellular pH support the above hypothesis. Incubation of the cells with NH_4Cl or CH_3NH_2 caused a significant increase in intracellular pH. The small magnitude of the increase was probably a consequence of the large buffering capacity of reticulocytes and erythrocytes due to their high content of haemoglobin. More significantly, there was a marked difference in the internal pH determined with [^{14}C]DMO from that obtained with $(^{14}CH_3)_2NH$, indicating pH heterogeneity within the cells [21—24]. Previous investigations of mature erythrocytes demonstrated no such differemce, from which it was concluded that the interior of these cells is homogeneous [16,24]. In the case of reticulocytes, pH heterogeneity is probably due to the presence of intracellular organelles such as mitochondria and intracellular vesicles which are absent in mature erythrocytes. The results obtained with $(^{14}CH_3)_2NH$ indicate that at least one intracellular component has a pH as low as, or even lower, than pH 7. This is unlikely to be the mitochondria which are believed to have a high internal pH [24]. More likely it is the intracellular vesicles.

Because of the heterogeneity of the cells it is not possible to calculate the exact pH of any individual cell component. However, it is reasonable to conclude that least one intracellular component, probably some or all of the vesicles, has a low pH and that the pH of this component is elevated to a greater degree than is the pH of more alkaline parts of the cell during the incubation with NH_4Cl and CH_3NH_2. Moreover, the pH of this component in control cells is probably lower and the degree of elevation of the pH produced with NH_4Cl or CH_3NH_2 is probably greater than indicated by the pH measurements made with $(^{14}CH_3)_2NH$ because less than 50% of the cells were reticulocytes. The presence of mature erythrocytes would result in a higher mean intracellular pH of the total cell population as determined with $(^{14}CH_3)_2NH$ and would

dampen the apparent change in pH of an acidic cell organelle present in only a fraction of the cells. Another problem in the interpretation of data such as those obtained in the present work is that the number of H^+ present in small intracellular vesicles may be extremely low. As was pointed out by Caldwell [21], under such circumstances, the term pH does not have its usual meaning of H^+ activity at a given instant in time. Instead, it represents the average H^+ activity which is present over a longer period.

The question must be raised as to whether the intracellular vesicles of reticulocytes are lined by membranes which have properties similar to those of the lysosomes found in other types of cells. Possibly, some reticulocyte vesicles are derived from lysosomes. Cellular maturation may have been accompanied by loss of the distinguishing morphological features of lysosomes without loss of the capacity to concentrate H^+. If this is so, the process of iron uptake by immature erythroid cells may involve endocytosis of the transferrin-iron complex, fusion of the endocytotic vesicles with lysosomes or lysosome-like organelles, iron release from transferrin as a consequence of protonation, and release of transferrin from the cell by exocytosis of those parts of the membrane of the combined intracellular vesicle which contain the transferrin receptors.

Two other observations on cellular uptake of transferrin-bound iron should be considered in relationship to the present work. Firstly, the only other documented situation in which reticulocyte uptake of iron is inhibited but that of transferrin enhanced is that due to the exposure of the cells to hemin [39]. In this situation, there appears to be a block to iron release from transferrin after it has been taken up by the cell. Whether the mode of action of hemin is similar to that of weak bases is yet to be established. The other observation is that CH_3NH_2 reduced the rate of iron uptake by cultured rat fibroblasts [40]. The mechanism of iron uptake by these cells is not known but it is possible that iron release from transferrin occurs within lysosomes after endocytosis of the protein-iron complex. As mentioned above, it is well known that weak bases can raise intralysosomal pH, an effect which would be expected to inhibit iron release from the protein it it were present in these organelles.

Two other explanations for the results presented in this paper were considered. Firstly, there was the possibility that the inhibition of iron uptake produced by NH_4Cl and the weak bases was a consequence of a fall in the cellular levels of ATP. Direct measurement showed that this was not the case (Table IV). Alternatively, elevation of intracellular pH could conceivably lead to increased breakdown of NADH, decreased NADH-dependent methaemoglobin reduction, increased methaemoglobin levels and an increase in the free haem pool in the cells since the affinity of haem for globin is less in methaemoglobin than in haemoglobin. The free haem would inhibit iron uptake by the cells, as mentioned above. However, no elevation of methaemoglobin level was observed (Table IV). Hence, this is unlikely to be the correct explanation for the observed results.

In summary, it was found that certain weak bases such as NH_3 (derived from NH_4Cl) and CH_3NH_2 inhibit the uptake of transferrin-bound without inhibiting the interaction of transferrin with its receptor or the uptake of the protein by the cell. Hence, these reagents act on the process by which iron is released from the transferrin molecule. The results support the hypothesis that this occurs

within endocytotic vesicles as a result of protonation of transferrin and that the bases inhibit iron release by reacting with vesicular H^+. Further work is required to verify this hypothesis and to determine the source of H^+ and how iron released from transferrin is transported across the membrane of the intracellular vesicles.

Acknowledgements

The author is indebted to Miss O. Siira for technical assistance. The work was supported by research grants from the Australian Research Grants Committee and the University of Western Australia.

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