Biochimica et Biophysica Acta, 464 (1977) 389—398

BBA 77582

IRON UPTAKE BY IMMATURE ERYTHROID CELLS

MECHANISM OF DEPENDENCE ON METABOLIC ENERGY

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(Received June 29th, 1976)
(Revised manuscript received September 17th, 1976)

Summary

The mechanism by which the utilization of transferrin-bound iron is linked with cellular metabolism was investigated using rabbit reticulocytes and bone marrow cells. The rate of metabolism was altered by the use of inhibitors which act at different sites in the metabolic pathway (NaF, sodium fluoroacetate, rotenone, 2,4-dinitrophenol, NaCN) and by the addition of metabolic substrates (inosine, sodium pyruvate, sodium lactate). Measurements were made of the rates of iron and transferrin uptake and, in many of the experiments, of cellular ATP and NADH concentrations. The results showed that there was a significant correlation between the rate of iron uptake and the ATP concentration of the cells, but no correlation was found with the NADH concentration. The rate of transferrin uptake was inhibited to a lesser degree than that of iron uptake, and only when the ATP concentration had fallen below that necessary to inhibit iron uptake. It is concluded that the rate of uptake of transferrin-bound iron by immature erythroid cells is dependent on the intracellular concentration of ATP but is independent of the NADH concentration.

Introduction

Immature erythroid cells are able to take up transferrin-bound iron and utilize it for haemoglobin synthesis [1,2]. It is recognized that the steps in this process are dependent on a supply of metabolic energy [2—4]. However, the mechanism by which utilization of transferrin-bound iron is linked with cellular metabolism is not known. At least three possibilities must be considered, viz. (1) the process is dependent on the intracellular concentration of ATP, (2) there is a direct link between iron utilization and mitochondrial oxidative meta-

bolism and (3) NADH forms the link between cell metabolism and iron uptake [4,5].

The experiments described in this paper were designed to investigate the three possibilities listed above. Cellular metabolism was altered by using different metabolic inhibitors and metabolic substrates in order to ascertain the steps of intermediary metabolism upon which the uptake of transferrin-bound iron was dependent. Cellular concentrations of ATP and NADH were measured in serveral of the experiments so that any correlation between the concentrations of these substances and iron uptake could be evaluated. The results indicate that the uptake of transferrin-bound iron by rabbit reticulocytes and bone marrow cells is dependent on the concentration of ATP in the cells but is independent of NADH concentration and is not directly linked with mitochondrial function.

Materials and Methods

Materials. Iron-59 ($FeCl_3$, 10—20 μCi/μg) and iodine-125 (NaI, carrier-free) were purchased from the Radiochemical Centre, Amersham, England. NaCN was obtained from B.D.H. Ltd., Poole, England. The other metabolic inhibitors, the metabolic substrates and the reagents used to assay ATP and NADH were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. With exception of rotenone, the solutions of inhibitors and substrates were prepared by dissolving the compounds in 0.15 M NaCl and adjusting the pH to 7.4 with 0.1 M HCl or 0.1 M NaOH. Rotenone was dissolved at high concentration in 95% ethanol and then diluted to the final concentration with 0.15 M NaCl. The rotenone was then in the form of an emulsion. Controls for the incubations in which rotenone was present contained the same concentration of ethanol as did the incubation mixtures which contained rotenone.

Purification and labelling of proteins. Rabbit transferrin was isolated and labelled with ^{125}I and ^{59}Fe as previously described [6,7]. In some experiments the transferrin present in freshly obtained rabbit plasma was labelled with ^{59}Fe by addition of the radioiron as its complex with nitrilotriacetic [8], care being taken that the latent iron-binding capacity of the plasma was not exceeded.

Reticulocytes and bone marrow cells. Reticulocytes were obtained from rabbits with phenylhydrazine-induced haemolytic anaemia [8]. The cells were washed three times in ice-cold 0.15 M NaCl and then suspended in Hanks and Wallace balanced salt solution [9]. The reticulocyte counts of the cells used in the present experiments varied from 32 to 95%. Bone marrow cells were obtained from the long bones of normal, untreated rabbits. They were washed and suspended in Eagle's medium [10] containing 1% bovine serum albumin as described previously [11].

Incubation procedure. All incubations were performed at 37° C. The methods used to incubate the cells and to collect and wash samples for counting of radioactivity were the same as in earlier work [11]. In some experiments the inhibitors were added to the cells at the same time as the transferrin labelled with ^{59}Fe and ^{125}I or plasma labelled with ^{59}Fe. In other experiments the cells were preincubated with the inhibitors and/or substrates at 37° C before the labelled

transferrin or plasma was added. The preincubation time was 60 min unless otherwise stated.

Analytical methods. Iron concentration was measured by the procedure recommended by the International Committee for Standardization in Haematology [12]. The total iron-binding capacity of solution containing transferrin was determined by the method of Morgan and Carter [13]. Reticulocytes were counted on dry smears after staining with new methylene blue. Cell samples were deproteinized with $HClO_4$ [14] and ATP was then measured by a standard method [15] which utilizes hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1) and glucose-6-phosphate dehydrogenase D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49). For the estimation of NADH, samples were removed from the incubation mixture and processed and assayed according to the method of Burch [16].

Radioactivity was measured in a three-channel well-type scintillation counter. The rate of iron uptake was calculated from the linear regression of at least four values obtained over a 30 min incubation period. Three aspects of transferrin uptake [4] were determined, (1) *adsorption*, the amount of transferrin bound to the cells immediately after mixing with them at 4°C, (2) *rate of uptake*, the initial rate of uptake of the protein by the cells during the first 2 min of incubation at 37°C and (3) *amount of uptake*, the difference between the quantity of transferrin on the cells when the plateau phase of uptake was reached (after 10—15 min incubation at 37°C) and that resulting from adsorption.

Results

Changes in rate of iron uptake and cellular ATP concentration

Bone marrow cells or reticulocytes were incubated from zero time with the inhibitors and ^{59}Fe-labelled plasma. Iron uptake and ATP levels were measured during a 30 min period of incubation. The inhibitors used were NaCN (50 mM) 2,4-dinitrophenol (2.5 mM), rotenone (0.01 mM), NaF (25 mM) and sodium fluoroacetate (50 mM). The results obtained with NaCN are shown in Figs. 1 and 2. Iron uptake by bone marrow cells (Fig. 1) and by reticulocytes (Fig. 2) was the same in the presence of NaCN as in its absence during the first 5 min of incubation. This was followed by a marked reduction in the rate of iron uptake during the subsequent period of incubation. Cellular ATP levels began to fall immediately after commencing the incubations and had reached low levels by the time the rate of iron uptake had diminished. The pattern of changes found with the other inhibitors was the same as with NaCN. In all cases the rate of iron uptake did not commence to fall until after 4—6 min incubation and in this time cellular ATP values for bone marrow cells declined to 30% or less of the control values and for reticulocytes had decreased to 40—60% of the controls.

Effects of metabolic inhibitors and substrates

The role of cellular metabolism in iron and transferrin uptake was investigated by incubating reticulocytes with the inhibitors and/or substrates for 1 h at 37°C and then incubating with ^{125}I- and ^{59}Fe-labelled transferrin. In the first

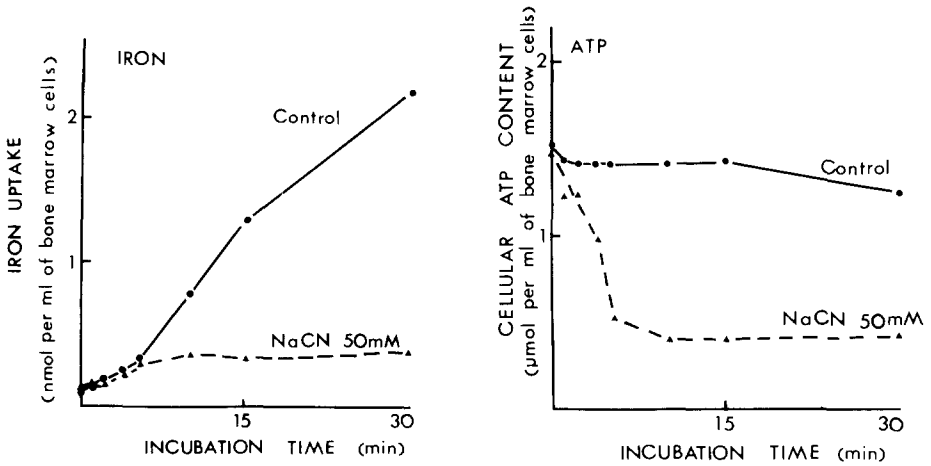


Fig. 1. Effect of NaCN (50 mM) on iron uptake and cellular ATP concentration in rabbit bone marrow cells incubated at 37°C. The iron and transferrin concentrations of the incubation solution were 0.009 and 0.010 mM, respectively.

series of experiments the substrates, inosine, sodium lactate and sodium pyruvate were used in combination with NaCN. The results are summarized in Table I. NaCN (50 mM) inhibited the rates of uptake of both transferrin and iron, but iron uptake was inhibited to a greater degree. Each of the substrates when used alone stimulated the rate of iron uptake but had no effect on the rate of transferrin uptake. Neither NaCN nor the substrates had a significant effect on transferrin adsorption or the amount of transferrin uptake.

Cyanide-induced inhibition of iron uptake was prevented when inosine was also present in the incubation mixture. In addition, the rate of transferrin

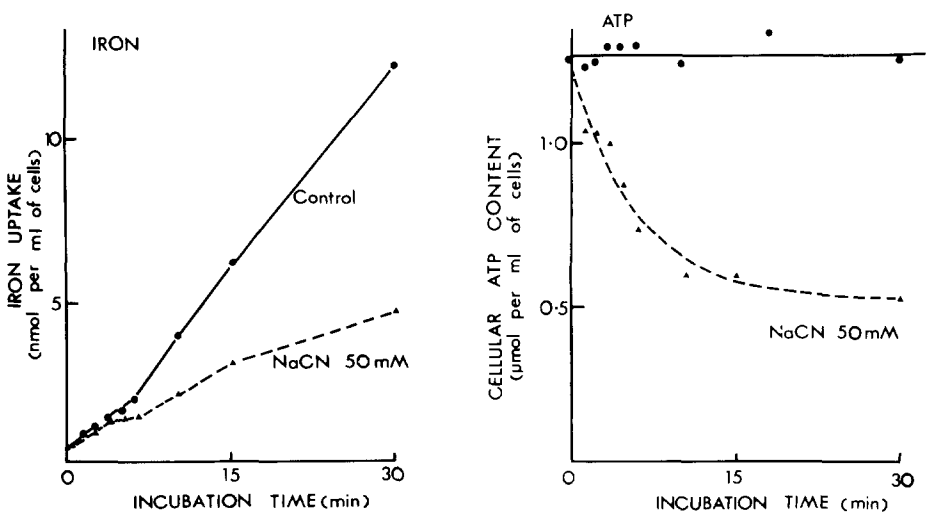


Fig. 2. Effect of NaCN (50 mM) on iron uptake and cellular ATP concentration in rabbit reticulocytes incubated at 37°C. The iron and transferrin concentrations of the incubation solution were 0.022 and 0.031 mM, respectively. The reticulocyte count was 78%.

TABLE I

EFFECT OF INOSINE, LACTATE, PYRUVATE AND NaCN ON IRON AND TRANSFERRIN UPTAKE

Reticulocytes were pre-incubated with the substances indicated for 60 min at 37°C. Transferrin labelled with ^{125}I and ^{59}Fe was then added and iron and transferrin uptake was measured during incubation at 37°C for 30 min. The concentrations used were: inosine, 10 mM; sodium lactate, 10 mM; sodium pyruvate, 10 mM; NaCN, 50 mM. The results are given as a percentage of the value obtained with control cells which were incubated without the additions shown in the table. Each value is the mean ± S.E. of four measurements or the mean and range of values (in parentheses) of two measurements made in separate experiments.

Substance added	Percent control value		
	Rate of iron uptake	Rate of transferrin uptake	Total transferrin uptake
Inosine	141 ± 9.8	104 ± 2.5	94 ± 1.0
Lactate	137 ± 7.4	105 ± 2.7	103 ± 1.5
Pyruvate	111 (106, 117)	95 (92, 98)	100 (95, 105)
Cyanide	23 ± 2.0	63 ± 1.2	93 ± 2.6
Inosine and lactate	120 ± 4.5	109 ± 2.2	106 ± 1.5
Inosine and pyruvate	125 (125, 125)	117 (114, 120)	129 (121, 137)
Inosine and cyanide	86 ± 5.6	106 ± 4.7	106 ± 2.1
Lactate and cyanide	16 ± 3.2	77 ± 2.1	92 ± 2.1
Pyruvate and cyanide	34 (24, 44)	49 (30, 70)	80 (76, 84)

uptake was returned to the control level. By contrast to the effects of inosine, neither sodium lactate (10 mM) nor sodium pyruvate (10 mM) had any effect on the inhibition of the rate of iron uptake produced by NaCN.

Measurements of cellular ATP and NADH concentrations were made in one experiment in which reticulocytes were preincubated with different combinations of NaCN, inosine and sodium lactate prior to incubation with labelled transferrin (Table II). Incubation with NaCN caused a fall in both ATP and NADH levels. However, while the ATP concentration fell to 15% of the con-

TABLE II

EFFECT OF CYANIDE, INOSINE AND LACTATE ON CELLULAR ATP AND NADH CONCENTRATIONS AND IRON AND TRANSFERRIN UPTAKE

The concentrations of the added substances were: NaCN, 50 mM; inosine, 10 mM and sodium lactate, 10 mM. The cells (88% reticulocytes) were pre-incubated with the additions for 30 min before iron and transferrin uptake were measured. The results are given as percentage of control values obtained with cells which were incubated in the absence of the added substances. The cellular ATP and NADH values are the means of four determinations made on cell samples removed at 0, 12, 24 and 36 min during incubation with ^{59}Fe- and ^{125}I-labelled transferrin. The mean ATP and NADH concentrations of the control cells were 2.46 and 0.12 μmol/ml cells, respectively. Each value given in the table is the mean ± S.E. of four measurements

Addition	Percent control value			
	ATP	NADH	Rate of iron uptake	Total transferrin uptake
Cyanide	15 ± 0.3	73 ± 3.1	11 ± 3.5	79 ± 3.3
Inosine	120 ± 5.7	110 ± 3.3	105 ± 4.1	96 ± 7.0
Inosine and cyanide	41 ± 0.9	68 ± 1.7	52 ± 5.4	115 ± 7.0
Lactate	93 ± 2.2	103 ± 2.4	112 ± 5.1	105 ± 4.9
Lactate and cyanide	17 ± 1.3	74 ± 1.3	13 ± 4.5	97 ± 6.4
Inosine and lactate	113 ± 3.0	85 ± 1.4	103 ± 4.2	109 ± 7.1

trol value, that of NADH decreased to only 75% of the controls. With inosine there was an increase in both cellular ATP and NADH levels. In the presence of inosine plus NaCN, ATP levels increased to nearly three times the values obtained with NaCN alone. However, there was no change in the cellular NADH concentration. When sodium lactate was used in combination with NaCN the ATP and NADH values were similar to those found with NaCN alone. The effects of the various treatments on the rate of iron uptake were generally similar to those on ATP concentrations but appeared to be unrelated to NADH concentrations. However, it should be noted that inosine produced a relatively greater increase in ATP concentration than in rate of iron uptake, while lactate decreased ATP levels slightly but increased iron uptake.

The relationship between the cellular ATP and NADH concentrations and iron and transferrin uptake by reticulocytes was further investigated using different concentrations of rotenone. The results are summarized in Table III. The ATP concentrations fell progressively as the rotenone concentration was increased from 0.00001 to 0.1 mM. By contrast, cellular NADH levels increased. The rate of iron uptake remained relatively constant until the rotenone concentration was raised to between 0.0005 and 0.005 mM but then decreased to reach a value of only 8% of the control when 0.1 mM rotenone was used. There was little inhibition of transferrin uptake except at the highest concentration of rotenone.

Relationship of cellular ATP and NADH concentration and rate of iron uptake

The data on the rate of iron uptake and cellular ATP levels from eleven experiments with reticulocytes and four experiments with bone marrow were analysed. The values for the rate of iron uptake, expressed as a percentage of control values, were plotted against the values for cellular ATP concentration, also expressed as a percentage of controls. There was a highly significant correlation between the rate of iron uptake and ATP concentration, both for reticulocytes (Fig. 3) and bone marrow cells (Fig. 4).

TABLE III

THE EFFECT OF PRE-INCUBATION WITH ROTENONE ON CELLULAR ATP AND NADH CONCENTRATIONS AND IRON AND TRANSFERRIN UPTAKE IN RETICULOCYTES

The cells (78% reticulocytes) were pre-incubated with rotenone at 37°C for 15 min before samples were taken for estimation of ATP and NADH. Iron and transferrin uptake was then measured. The cellular ATP and NADH concentrations in control cells pre-incubated for 15 min in the absence of rotenone were 2.72 and 0.14 μmol/ml cells, respectively.

Rotenone concentration (mM)	Percent control value					
	ATP	NADH	Iron uptake		Transferrin uptake	
			Rate	Rate	Amount	Total
0.00001	89	113	100	100	96	100
0.00005	81	110	97	96	105	100
0.0005	68	164	96	104	115	106
0.005	43	174	56	103	118	108
0.01	42	188	50	100	118	109
0.1	40	205	8	44	89	66

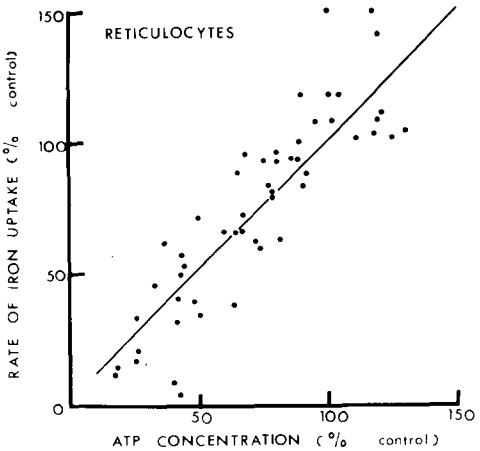
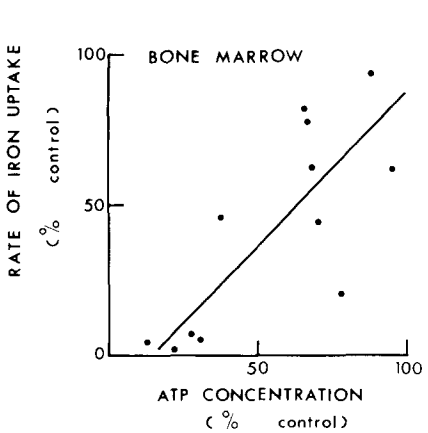


Fig. 3. Relationship between rate of iron uptake and cellular ATP concentration in rabbit reticulocytes. The ATP concentration was altered by pre-incubating the cells with metabolic substrates or inhibitors as described in the text. The equation of the regression line is $y = 0.97x + 3.2$ ($r = 0.87$, $P < 0.005$).



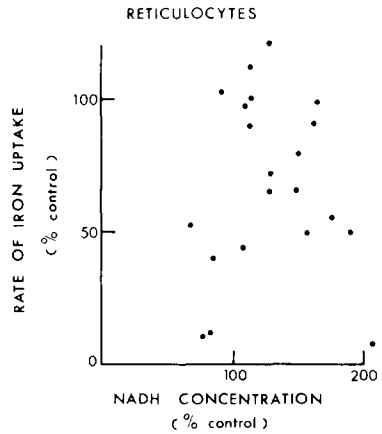


Fig. 4. Relationship between rate of iron uptake and cellular ATP concentration in rabbit bone marrow cells. The ATP concentration was altered by pre-incubating the cells with metabolic substrates or inhibitors. The equation of the regression line is $y = 1.02x - 14.7$ ($r = 0.80$, $P < 0.01$).

Fig. 5. Relationship between rate of iron uptake and cellular NADH concentration in rabbit reticulocytes pre-incubated with various metabolic substrates and inhibitors.

Data on iron uptake rates and cellular NADH levels obtained in five reticulocyte experiments were analysed in a similar manner. However, no significant correlation was found (Fig. 5).

Discussion

The results presented in this paper show that the uptake of transferrin-bound iron by immature erythroid cells is closely correlated with the intracellular concentration of ATP. Such a correlation suggests, but does not prove, that iron uptake is dependent on cellular ATP levels. The results do not support the

hypotheses that iron uptake is directly linked with the mitochondrial electron chain [4] or with the function of a NADH + H^+ coenzyme [5].

Several lines of evidence indicate the importance of ATP levels. Firstly, when reticulocytes or bone marrow cells were incubated simultaneously with ^{59}Fe-labelled transferrin and metabolic inhibitors the rate of iron uptake did not start to decrease until 5—10 min had elapsed, by which time the ATP concentrations had fallen appreciably. Secondly, the rate of iron uptake by reticulocytes was significantly elevated when they were incubated with inosine which also elevated cellular ATP levels. Inosine, too, was able to prevent the inhibition of iron uptake produced by NaCN and this was also accompanied by an elevation of ATP concentration. The two other substrates used in combination with cyanide, sodium lactate and sodium pyruvate, were without effect on cyanide-inhibited iron uptake and on the depressed ATP values found in the cyanide-treated cells. The third line of evidence implicating ATP as the link between cellular metabolism and iron uptake is the significant direct correlation found between the rate of iron uptake and ATP concentrations of reticulocytes (Fig. 3) and bone marrow cells (Fig. 4).

A closer correlation between the rate of iron uptake and cellular ATP levels was found for reticulocytes ($r = 0.873$, $P < 0.005$) than for bone marrow cells ($r = 0.799$, $P < 0.01$). This probably resulted from the fact that considerably more measurements were made with reticulocytes than with bone marrow and, more particularly, because the reticulocytes consisted of a much more homogeneous cell mixture than did the marrow cells. Nearly all of the cells in the reticulocyte-rich blood were reticulocytes. Hence, they would nearly all be able to take up transferrin-bound iron and be affected in a similar manner by the inhibitors and substrates used. The bone marrow, on the other hand, was from normal rabbits in which only about 25% of the cells are immature erythroid cells and capable of taking up iron. The metabolic inhibitors and substrates would be expected to affect the metabolism of both erythroid and non-erythroid cells and in the latter types of cells produce changes in ATP concentration without affecting iron uptake.

As a result of an earlier study on the effects of a series of metabolic inhibitors on iron and transferrin uptake by reticulocytes it was proposed that iron uptake is closely linked to the mitochondrial respiratory chain and is not simply dependent on respiration for a supply of ATP [4]. However, this hypothesis does not stand up to the more detailed scrutiny provided by the present investigation. The close correlation between the rate of iron uptake and ATP levels has been discussed above. Moreover, inhibitors of glycolysis (NaF) and of the citric acid cycle (sodium fluoroacetate) blocked iron uptake (and also lowered ATP concentrations) in like manner to inhibitors of mitochondrial function. Furthermore, restoration of cellular ATP values by incubation with inosine in the presence of NaCN, returned the rate of iron uptake almost to the control value even though mitochondrial electron transfer was still blocked by the NaCN. Finally, dinitrophenol which uncouples oxidative phosphorylation and lowers cellular ATP levels does not inhibit mitochondrial electron transfer. However, it is an effective inhibitor of iron uptake by reticulocytes and bone marrow cells.

The mechanism by which inosine raises the ATP concentration of erythrocytes has been investigated only in mature cells. After entry into the cell the

inosine undergoes phosphorolytic cleavage to hypoxanthine and ribose 1-phosphate which in turn is metabolized to glyceraldehyde 3-phosphate. Each mol of glyceraldehyde 3-phosphate can then be converted to pyruvate yielding 2 mol of ATP in the process [17,18]. Energy production in the reticulocyte occurs principally by oxidative phosphorylation and to a less extent by substrate level phosphorylations in the Embden-Myerhof pathway [19]. However, it is likely that energy production from inosine occurs by the same mechanism in reticulocytes as in mature erythrocytes. Hence, when oxidative phosphorylation is inhibited, as by the use of NaCN, ATP production from inosine can still proceed and prevent the cellular concentration of ATP from falling to levels as low as those observed when the inhibitor of oxidative phosphorylation is used alone (Table II). By contrast to inosine, both pyruvate and lactate are metabolized after the energy-yielding steps of the Embden-Myerhof pathway. This provides an explanation for the observation that these compounds did not elevate ATP concentration or the rate of iron uptake when used in the presence of NaCN. It should be noted that a very high concentration (50 mM) of NaCN was used in the present experiments. This concentration was chosen because it was found in previous work that 25—50 mM NaCN was required to produce approx. 80% inhibition of iron uptake by rabbit reticulocytes [4] and it was desired to produce a large degree of inhibition.

The rate of iron uptake by reticulocytes did not appear to be correlated with cellular NADH concentrations in the experiments performed in the present investigation (Fig. 5). For instance, iron uptake was inhibited in the presence of elevated (Table III) or slightly depressed (Table II) NADH values, and the addition of inosine to a NaCN-containing incubation mixture elevated iron uptake without altering the NADH level (Table II). Hence, the cellular concentration of NADH does not appear to act as a rate-limiting factor for iron uptake by reticulocytes. This, plus the evidence discussed above, appears to exclude the hypothesis presented by Egyed [5], that iron uptake by reticulocytes is independent of the ATP concentration but is dependent on an enzyme function with a $NADH + H^+$ coenzyme which detaches iron from transferrin. It is more likely that the detachment of iron from transferrin is in some way dependent on ATP and that this is the rate-limiting step in iron uptake. Egyed [20] has recently shown that ATP can release iron from transferrin in a cell-free in vitro system. However, the mechanism of ATP involvement in the cell remains to be elucidated. These considerations do not rule out the possibility that NADH is also involved in a step subsequent to that of ATP in the intracellular metabolism of iron (e.g. reduction to the ferrous form) but this step is not rate limiting.

The metabolic inhibitors used in the present work affected the rate of iron uptake to a greater degree and/or at a lower inhibitor concentration than they affected the rate of transferrin uptake. Hence, in the presence of metabolic inhibitors the rate of iron uptake is limited by the rate of detachment of iron from transferrin or the rate of passage through a subsequent portion of its metabolic pathway rather than by the rate of uptake of its transport protein. The rate of uptake of transferrin was inhibited only under conditions in which the cellular ATP concentration was reduced to very low levels. Possibly, transferrin uptake, like iron uptake, is an ATP-dependent process, but is only limited

by the ATP concentration when this has fallen to a value considerably below that which leads to a decrease in iron uptake. Transferrin uptake by reticulocytes may occur by endocytosis [21], a process which has been shown to be dependent on cellular metabolism in many other types of cells [22].

Acknowledgements

This work was supported by grants from The Australian Research Grants Committee and The University of Western Australia.

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