Regulation of Iron Entry into Reticulocytes. I. Feedback Inhibitory Effect of Heme on Iron Entry into Reticulocytes and on Heme Synthesis

By P. Ponka and J. Neuwirt

For several years past some mechanisms of the delivery of iron to the immature red blood cell have been elucidated. It is now well known how the factors of the external environment, i.e., saturation of transferrin, the amount of the iron-transferrin complex and the distribution of iron on the molecule of transferrin influence the rate of iron entry into erythroid cells. Transferrin carrying iron is bound to the receptors on the membrane of immature red cells where it remains for about one minute and during this interval iron is removed by a process which involves ATP and ascorbic acid. The affinity of transferrin for reticulocytes is up to 100 times greater than for mature erythrocytes and iron uptake gradually decreases with maturation.

Dependence of the iron entry into the cell on heme and hemoglobin concentration inside the cell or on the rate of their synthesis is not yet properly known. Katz deduces from experiments with lead which inhibits the formation of heme and does not interfere with iron uptake, that the rate of iron uptake is not controlled by the rate of globin synthesis per se. On the other hand in the presence of inhibitors of globin synthesis a diminished iron uptake was observed.

In our experiments we followed iron incorporation into reticulocytes during heme synthesis inhibited by heme, lead or isonicotinic acid hydrazide and during globin synthesis inhibited by cycloheximide. Our results show that iron does not enter the cell independently of heme synthesis and that there exists a feedback mechanism by means of which heme or globin prevents surplus accumulation of iron inside the cell.

Methods

Experiments were carried out on rabbit reticulocytes. Reticulocytosis was produced by removing 15 ml. of blood per Kg. of weight from the ear vein in three consecutive days. On the fifth day rabbits were exsanguinated (arteria carotis) under ether narcosis. The
blood rich in reticulocytes (usually 10–15 per cent) was collected into heparin and red
cells were washed three times by NKM solution according to Borsook.17

Five ml. of packed cells were incubated as a 10 per cent suspension in the commercial
medium (SEVAC, Prague) containing one third of fresh rabbit plasma. The composition
of this medium18 with respect to amino acids, salts and co-factors is practically the same
as that described by Morgan, Morton and Parker.19 The pH of the medium was adjusted
to 7.4 with 7.5 per cent NaHCO₃ before the plasma was added. The complete medium
was preincubated with ⁵⁹Fe approximately one hour before the beginning of the incuba-
tion to enable the radioiron to bind itself to transferrin. The iron content20 and the iron
binding capacity of the plasma2¹ were determined according to Ramsay. The average level
of the plasma iron was 154 ± 35 µg per cent and the total iron binding capacity was
351 ± 102 µg per cent. From the value of specific activity of ⁵⁹Fe, which is mentioned
below, it is evident that added radioiron can increase the concentration of iron in the
medium by about 1 per cent. In spite of this we are convinced that radioiron does not
exceed the iron binding capacity of the plasma.

The amount of ⁵⁹Fe (obtained from Zentralinstitut für Kernforschung, Dresden, specific
activity 10mC/1 mg. Fe) used was 1.5 µC per 50 ml. of the total incubation mixture.

The incubation was carried out under moistened air at 37 C. in a shaker. Samples were
drawn at different time intervals and placed into centrifuge tubes containing 1 ml. of 1
per cent KCN. The tubes were kept under refrigeration. The cells were washed in
isotonic saline at least four times and then were divided into two parts. From one hemin
was crystallized while the other was used for the determination of reticulocyte radioactivity.
0.2 ml. of washed cells were resuspended in saline to a final volume of 0.5 ml. After
determination of activity in the well type scintillation counter (Tesla NRQ 612 1), the
amount of hemoglobin (in g per cent) was measured according to Drabkin.

Hemin was isolated from reticulocytes according to Labbe and Nishida.2² No cold
hemoglobin was added prior to the isolation of hemin because the yield of hemin crystals
was sufficient varying around 1 mg. Hemin crystals were dissolved in 0.5 ml. of 0.02
N NaOH and their radioactivity measured under the conditions used for the measuring
of the radioactivity of the cells. The amount of hemin was determined spectrophotome-
trically. The total solution was transferred to graded tubes. 0.5 ml. 10 per cent KCN
solution was added and the volume of the solution in the tubes equalized by adding
distilled water.2³ Extinction of cyanhemine2⁴ was measured at 545 m on the spectro-
photometer Colleman Junior. The recovery of isolated heme from the reticulocytes varied
around 60 per cent. Specific activity was expressed in cpm per mg. of hemin.

In some experiments hemin was extracted from hemoglobin from which compounds of
low molecular weight had been separated by chromatography of Sephadex-G 25. Washed
reticulocytes were lysed by distilled water and by repeated freezing and thawing. The
stroma was removed by centrifugation at 20,000 × g. for 30 min. The supernatant was
chromatographed on a Sephadex G-25 column equilibrated with 0.05 M phosphate buffer,
pH 8.0. Hemoglobin was eluted with the same buffer and precipitated by adding enough
20 per cent trichloracetic acid to give a final concentration of approximately 7 per cent.
After centrifugation the precipitated hemoglobin was subjected to the extraction procedure
usually used for the isolation of heme from reticulocytes. The hemin was further treated
as described above. This procedure removes free heme which may be present in hemo-
lysate and which is not bound to hemoglobin.

The percentage of the total radioiron in the cell which is present in heme was estimated
for each time interval from the radioactivity of whole cells, the amount of hemoglobin
in these cells and the specific activity of heme according to the formula:

$$\frac{H \cdot V \cdot C_{Hb} \cdot W_{H}}{A \cdot W_{Hb}} \times 4000 \text{ (in per cent),}$$

where $V$ = volume of suspension of reticulocytes in ml., $A$ = radioactivity of this sus-
pension, $C_{Hb}$ = gram per cent of hemoglobin of this suspension, $H$ = specific activity
of hemin (in cpm. per mg. of hemin), $W_{H}$ = molecular weight of heme, $W_{Hb}$ = molecular
weight of hemoglobin. This formula is simplified if \( V = 0.5 \text{ ml.} \) and \( W_{\text{HB}} = 57 \text{ 412} \) (for rabbit = 25):

\[
\frac{H \cdot C_{\text{HB}}}{A} \times 21.42 \%.
\]

The calculated values indicate the radioiron distribution in the heme and nonheme pools of reticulocytes at different time intervals and it is denoted as cellular distribution of radioiron (CDRI).

Objections could be raised that erroneous value of cellular distribution of radioiron is calculated if the pool of free heme is present in reticulocytes. Under conditions of normal globin synthesis this pool cannot be large because we failed to prove significant decrease of specific activity of heme after purification on Sephadex G-25 of hemolysate from reticulocytes incubated two hours with \(^{59}\text{Fe}\). If a great amount of labeled free heme is present in the hemolysate, then the specific activity of heme isolated from hemolysate would be greater than that isolated from hemoglobin purified on Sephadex G-25.

However, during inhibited globin synthesis, there is reportedly an accumulation of free heme (see below). We estimated the specific activity of heme which was isolated from whole reticulocytes so that value "\( H \)" includes both free and bound heme. The only value which could be affected is "\( C_{\text{HB}} \)" because the molar yield of hemoglobin is smaller if some formed heme is not bound in hemoglobin. Since the amount of hemoglobin formed during incubation of reticulocytes is negligible in comparison with the amount of hemoglobin which is already present in red cells, the value "\( C_{\text{HB}} \)" is not influenced by incubation. Consequently this value does not change in case of accumulation of free heme.

Highly labeled hemin was prepared from red blood cells taken from rats injected by 30-50 \( \mu \text{C} \) \(^{59}\text{Fe} \) 7 days before exsanguination. The erythropoiesis of rats was stimulated by bleeding to increase the utilization of the iron.

Hemin added to the medium was prepared from the rabbit blood according to Labbe and Nishida,22 recrystallized according to Shemin et al.26 and dissolved after Kanibian and London.27 Cycloheximide was the product of Up John (Actidione) and isonicotinic acid hydrazide (Nidrazid) obtained from Spofa (Prague).

RESULTS

Time Course of Cellular Distribution of Radioiron

At the beginning of our studies of the relation between uptake of radioiron by reticulocytes and its incorporation into the heme molecule, we attempted to express the relation quantitatively. A simple method was used which allowed determination of what percentage of the total iron which is incorporated into the reticulocytes appears in the heme. The cellular distribution of radioiron during the incubation of reticulocytes could thus be followed. From the Figure 1 it is evident that in normal reticulocytes only after 60 minutes, the radioiron is being equilibrated in the heme and nonheme pool of the cell (Solid line, Fig. 1). If heme synthesis is inhibited by some mechanism which does not affect the membrane transfer of iron or if heme synthesis is relatively lower than the iron entry into the cell, the curve will be located in the lower part confined by control curve (solid line) and abscissa.

Effect of Hemin on Iron Incorporation into Reticulocytes and on Heme Synthesis

Till now 11 different experiments have been carried out in which reticulocytes were incubated with hemin and with transferrin-bound radioiron. We preincubated reticulocytes with hemin for 5 to 30 minutes and measured the activity of intact reticulocytes and determined the specific activity of hemin...
from these reticulocytes over time intervals from 5 to 180 minutes. After addition of hemin (in concentrations from $10^{-4}$M to $3 \times 10^{-4}$M) to the incubation medium we found reduced reticulocyte radioiron uptake and a decrease in the incorporation of $^{59}$Fe into the heme in all experiments and in all time intervals. In more than one half of cases the percentile decreases in specific activities of hemes were greater or the same as the percentual decrease of $^{59}$Fe incorporation into reticulocytes after incubation with hemin. Using Wilcoxon's test we failed to find any statistical difference between percentage of decrease of iron incorporation into reticulocytes and heme in corresponding time intervals.

Results from one representative experiment are presented in Figure 2. This figure shows that the incorporation of iron into reticulocytes as well as into heme decreases with increasing concentration of hemin in the incubation medium. The evaluation of the relationship between iron entry into reticulocytes and heme in control and hemin incubated reticulocytes was made by determination of values of cellular distribution of radioiron. Figure 1 shows that the time course of curves in hemin treated and control reticulocytes is very similar indicating that hemin used in all three concentrations inhibits iron entry into the cell in proportion to the inhibition of its incorporation into heme.

Finally we made an experiment in which the statistical validity of values of cellular distribution of radioiron in control and hemin treated reticulocytes was tested. Reticulocytes were incubated in 40 separate flasks. One half con-
Fig. 2.—Effects of various concentrations of added hemin on the incorporation of $^{59}$Fe into reticulocytes and on the synthesis of heme. Cells had been preincubated 20 min. with hemin.

tained control medium while the other half contained hemin in a concentration of $3 \times 10^{-4}$ M. The incorporation of $^{59}$Fe into heme was depressed to the same extent as in intact reticulocytes after 30 or 60 minute incubation with hemin (to about 35 per cent). No statistical difference was found in the percentage of total cellular radioiron in heme between control and heme treated reticulocytes.

Two types of experiments were carried out in order to exclude some factors which influence the interpretation of the above mentioned results.

In one experiment we incubated 50 ml. of complete medium containing transferrin-bound radioiron and $10^{-4}$ M hemin for 90 minutes. Fifteen mg. of dissolved hemin was then added to the medium and the hemin extracted with acid acetone and crystallized. From $1.7 \times 10^{6}$ cpm. added as $^{59}$Fe only $2.7 \times 10^{3}$ cpm. appeared in hemin, i.e., less than 0.16 per cent of radioiron could leave the medium by exchange with unlabeled iron in hemin or by adsorption to hemin. This problem has not been investigated further since it was evident that the decrease in iron incorporation into reticulocytes in the presence of hemin cannot be explained by this phenomenon.

In the following series of experiments we studied the question whether hemin added to reticulocytes enters these cells. Reticulocytes were incubated in the usual medium which did not contain $^{59}$Fe. The highly radioactive hemin labeled with $^{59}$Fe was added to the incubation medium. We found that after a three-hour incubation with $10^{-4}$ M labeled hemin the radioactivity in reticulocytes corresponded to 4–5 $\mu$g. of hemin per 0.2 ml. of cells. The amount of hemin in reticulocytes increased with higher concentrations of hemin in the medium; the concentration of heme was no more than 10–12 $\mu$g. per 0.2 ml. of reticulocytes with a hemin concentration of $3 \times 10^{-4}$ M.

We found that approximately 10 $\mu$g. of hemin are incorporated per 100 mg.
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Table 1.—Effect of Lead on the Incorporation of $^{59}$Fe into Reticulocytes and Heme and on the Cellular Distribution of Radioiron (abbreviated CDRI)

<table>
<thead>
<tr>
<th>Time of incubation (min.)</th>
<th>Value</th>
<th>Control</th>
<th>$2 \times 10^{-4}$M lead acetate</th>
<th>$2.5 \times 10^{-4}$M lead acetate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cpm./0.2 ml. rtc</td>
<td>15,300</td>
<td>16,484</td>
<td>17,303</td>
</tr>
<tr>
<td></td>
<td>cpm./mg. heme</td>
<td>5,586</td>
<td>1,575</td>
<td>1,902</td>
</tr>
<tr>
<td></td>
<td>CDRI (%)</td>
<td>91.6</td>
<td>23.8</td>
<td>27.6</td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cpm./0.2 ml. rtc</td>
<td>22,868</td>
<td>17,523</td>
<td>22,827</td>
</tr>
<tr>
<td></td>
<td>cpm./mg. heme</td>
<td>8,470</td>
<td>1,930</td>
<td>2,125</td>
</tr>
<tr>
<td></td>
<td>CDRI (%)</td>
<td>92.9</td>
<td>28.3</td>
<td>24.4</td>
</tr>
<tr>
<td>180</td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

of hemoglobin after the three hour incubation of reticulocytes with $10^{-4}$M heme. This value we estimated by measuring the radioactivity of the hemoglobin after passing a hemolysate through Sephadex G-25.

Effect of Lead on Iron Incorporation into Reticulocytes and on Heme Synthesis

Table 1 represents the radioactivities of reticulocytes and the specific activities of hemes after 2 and 3 hours incubation with Pb$^{++}$ compared with the corresponding control values. A markedly inhibited heme synthesis and a normal or even higher $^{59}$Fe incorporation into whole reticulocytes results in accumulation of radioiron in the nonheme pool in the group containing lead.

Reticulocyte Radioiron Uptake in Heme Synthesis Inhibited by Isonicotinic Acid Hydrazide and by Hemin

In this series of experiments we followed incorporation of $^{59}$Fe into reticulocytes and heme in the presence of isonicotinic acid hydrazide (INH). Table 2 shows decrease in the incorporation of radioiron into heme during incubation of reticulocytes with INH while the entry of iron into such reticulocytes is not decreased but may even be increased. The statistical validity of the increased uptake of iron by the reticulocytes incubated with INH is demonstrated on Table 3. Results in this table were obtained after the 90 minute incubation of reticulocytes divided into 12 different control samples and 12 different samples containing $10^{-3}$M INH.

The results from another experiment are presented on Figure 3. If hemin is added to reticulocytes incubated with INH, the incorporation of iron into reticulocytes is decreased not only below the values found in the group with INH but even below control values. The incorporation of $^{59}$Fe into heme in reticulocytes incubated with INH is still further decreased after addition of hemin to the incubation medium (i.e., group with INH and hemin—Fig. 3), but the decrease of specific activities of heme is, under these circumstances, smaller than that of the iron entry into the cell. Intense accumulation of radioiron in the nonheme pool after incubation with INH (norm. 60–70 per cent in heme), which is evident from the time curve of cellular distribution of radioiron, is to a certain extent hindered by hemin (interrupted and dotted lines, Fig. 1).
Table 2.—Effect of Various Concentrations of INH and Various Time of Pre-incubation with INH on Incorporation of $^{59}$Fe into Reticulocytes and Heme. Recorded values are expressed as percentage of $^{59}$Fe incorporation into control reticulocytes and heme. R — % of incorporation into reticulocytes, H — % of incorporation into heme. The time of preincubation of corresponding control and INH treated cells were the same.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Time of preincubation with INH (min.)</th>
<th>Concentration of INH</th>
<th>Time of incubation/minutes/</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>$10^{-3}$M</td>
<td>R</td>
<td>108.9</td>
<td>113.9</td>
<td>123.1</td>
<td>107.5</td>
<td>116.5</td>
<td>135.3</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>61.1</td>
<td>76.0</td>
<td>84.4</td>
<td>87.0</td>
<td></td>
<td></td>
<td></td>
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<td>2</td>
<td>30</td>
<td>$10^{-3}$M</td>
<td>R</td>
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<td>139.8</td>
<td>151.0</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>57.0</td>
<td>81.3</td>
<td>85.2</td>
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</tr>
<tr>
<td>3</td>
<td>20</td>
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<td>R</td>
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</tr>
<tr>
<td></td>
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<td>H</td>
<td>67.9</td>
<td>79.7</td>
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</tr>
<tr>
<td>4</td>
<td>20</td>
<td>$2\times10^{-3}$M</td>
<td>R</td>
<td>107.3</td>
<td>114.6</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>24.6</td>
<td>31.2</td>
<td>37.6</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>$4\times10^{-3}$M</td>
<td>R</td>
<td>102.7</td>
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<td></td>
<td></td>
<td>H</td>
<td>7.5</td>
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<td>$8\times10^{-3}$M</td>
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<td>96.1</td>
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<tr>
<td></td>
<td></td>
<td>H</td>
<td>5.8</td>
<td>5.1</td>
<td>4.9</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>60</td>
<td>$10^{-3}$M</td>
<td>R</td>
<td>103.5</td>
<td>94.6</td>
<td>101.3</td>
<td>102.3</td>
<td>104.1</td>
<td>104.7</td>
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<td></td>
<td></td>
<td>H</td>
<td>82.1</td>
<td>66.4</td>
<td>71.1</td>
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<td>110.0</td>
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<tr>
<td></td>
<td></td>
<td>H</td>
<td>40.7</td>
<td>45.5</td>
<td>36.6</td>
<td>38.6</td>
<td></td>
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</tr>
</tbody>
</table>

Comparison of $^{59}$Fe Radioactivities in Whole Reticulocytes and in Those with Removed Stroma after Incubation with INH

We measured in control and INH treated reticulocytes the radioactivity of soluble fractions of hemolysate which were obtained after centrifugation of stroma at $20,000 \times g$ for 30 minutes. $^{59}$Fe radioactivity was measured in 0.5 ml. of hemolysate containing 0.2 ml. of reticulocytes, and we determined the hemoglobin content in each sample according to Drabkin. Radioactivities of all samples were recalculated to the same amount of hemoglobin.

Table 4 shows that after incubation of reticulocytes with $10^{-3}$M INH there is the increased incorporation of radioiron into hemolysate. We measured also the radioactivity in intact cells and we found that there is almost no difference between the radioactivity of intact cells and corresponding amount of hemolysate. If we express incorporation of $^{59}$Fe into heme after incubation with INH as a percentage of control values, we can see that with increasing time of incubation the difference between specific activities of hemin found in the control and in the INH treated reticulocytes is decreasing (the last column, Table 4). The same phenomenon can be observed in some experiments presented in Table 2.
This observation can be explained by a greater transfer of iron across the membrane when heme synthesis is inhibited. For this reason the specific activity of $^{59}$Fe in nonheme iron pool of reticulocytes incubated with INH is higher and relatively greater amount of radioiron enters heme. This possibility is further supported by the finding that the inhibition of $^{59}$Fe incorporation into heme depend on the time of preincubation of cells with INH (Table 2, Exp. No. 5). This is probably due to an accumulation of unlabeled iron in nonheme iron pool during preincubation of cells with INH. $^{59}$Fe added to cells later is diluted in this pool and less radioiron enters heme.

If greater concentrations of INH are used ($3-8 \times 10^{-3}$M, Figure 3, Table 2) the same extent of inhibition of $^{59}$Fe incorporation into heme may be observed during incubation. In this case, the inhibition of heme synthesis is probably very high.

**Iron Entry into Reticulocytes with Inhibited Globin Synthesis**

In this experiment we incubated reticulocytes with cycloheximide or hemin which were added to incubation medium at the same time as $^{59}$Fe. In Figure 4 it is shown that the amount of iron taken up by reticulocytes is, within the first...
Table 4.—Radioactivities in Stroma-Free Reticulocytes and Specific Activities of Hemes during Incubation with INH. INH and $^{59}\text{Fe}$ were Added to Cells Simultaneously.

<table>
<thead>
<tr>
<th>Time of incubation (minutes)</th>
<th>Radioactivity of reticulocytes (cpm./0.2 ml.)</th>
<th>Specific activity of heme (cpm./mg.)</th>
<th>s. a. heme ($%$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10 $\mu M$ INH</td>
<td>Control</td>
<td>10 $\mu M$ INH</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1677</td>
<td>1809</td>
<td>206</td>
</tr>
<tr>
<td>10</td>
<td>2713</td>
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<td>410</td>
</tr>
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<td>15</td>
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<td>60</td>
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<td>1490</td>
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<tr>
<td>120</td>
<td>16884</td>
<td>21480</td>
<td>4418</td>
</tr>
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</table>

Fig. 4.—Comparison of effects of cycloheximide and hemin on the reticulocyte radioiron uptake and on heme synthesis.

15 minutes of incubation with cycloheximide, entirely the same as in the control group, but is diminishing later. On the contrary though specific activity of heme decreases in cycloheximide-treated reticulocytes already in the first five minutes of incubation with an inhibitor of proteosynthesis. The situation appearing after addition of hemin is in agreement with the results found in the previous experiments and moreover it even resembles the pattern of iron incorporation into reticulocytes and heme described in the group with cycloheximide.

In this experiment we also determined values of cellular distribution of radioiron in all groups. In order to make changes in these values during incubation with cycloheximide or hemin more obvious, the values of cellular distribution of radioiron in experimental groups were expressed as percentage...
Table 5.—Changes in Percentage of Radioiron in Heme during Incubation of Reticulocytes with Hemin or Cycloheximide in Comparison with Controls. (CDRI is abbreviation of cellular distribution of radioiron.)

<table>
<thead>
<tr>
<th>Time of incubation (min.)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>120</th>
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</thead>
<tbody>
<tr>
<td>CDRI&lt;sub&gt;hemin&lt;/sub&gt;</td>
<td>100 (%)</td>
<td>&gt;100</td>
<td>93.8</td>
<td>80.5</td>
<td>96.8</td>
<td>93.0</td>
</tr>
<tr>
<td>CDRI&lt;sub&gt;control&lt;/sub&gt;</td>
<td>100 (%)</td>
<td>56.3</td>
<td>60.6</td>
<td>67.0</td>
<td>71.2</td>
<td>73.0</td>
</tr>
</tbody>
</table>

Fig. 5.—Incorporation of <sup>59</sup>Fe into cycloheximide treated reticulocytes and into reticulocytes treated with inhibitors of both heme and globin synthesis (INH and cycloheximide).

Fig. 5 shows the incorporation of <sup>59</sup>Fe into reticulocytes treated with cycloheximide and inhibitors of both heme and globin synthesis (INH and cycloheximide).

EFFECT OF HEME ON ERYTHROID MEMBRANE

of control values for each time interval (Table 5). Values approaching 100 percent show that in the experimental group relation between iron uptake by reticulocytes and its incorporation into heme is similar to that in the control group. Lower values indicate relative inhibition of heme synthesis and are suggestive of the accumulation of excess iron in the nonheme pool.

An Attempt to Influence the Reduced Iron Entry into Reticulocytes with Inhibited Proteosynthesis by Simultaneous Inhibition of Heme Synthesis

From Figure 5 it follows that INH partially removes the inhibitory effect of cycloheximide on iron entry into reticulocytes. This effect of INH is concentration dependent. Employed concentrations of INH were also added to control reticulocytes, but under these conditions an increased incorporation of <sup>59</sup>Fe into reticulocytes was observed only when the highest concentration of INH was used (5 × 10⁻⁴M). While INH in this concentration stimulated
Table 6.—Values of 15-minute Radioiron Incorporation into Heme (cpm./mg.) of Control Reticulocytes and of Reticulocytes Incubated with Cycloheximide and of Reticulocytes Incubated with both Cycloheximide and Various Concentrations of INH

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>$2 \times 10^{-4}$M Cycloheximide</th>
<th>$1.25 \times 10^{-4}$M INH</th>
<th>$2.50 \times 10^{-4}$M INH</th>
<th>$5.00 \times 10^{-4}$M INH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocytes</td>
<td>1595</td>
<td>616</td>
<td>521</td>
<td>379</td>
<td>152</td>
</tr>
</tbody>
</table>

the reticulocyte uptake of radioiron by 15–20 per cent, almost 100 per cent stimulation of $^{59}$Fe incorporation was observed after addition of the same concentration of INH to the group with cycloheximide.

Values of heme synthesis from this experiment are demonstrated in Table 6. The addition of INH together with cycloheximide reduces incorporation of iron into heme to a greater extent than cycloheximide by itself.

In this experiment cells of all groups had been preincubated for 30 minutes before the label was added.

**DISCUSSION**

Studying the relations between the incorporation of iron into cells and heme, we determined the percentage of radioiron in heme from the total radioiron, which had entered the cell. The time course of this cellular radioiron distribution proves the presence of a nonheme iron pool which results in a certain time delay before $^{59}$Fe enters the hemin molecule (Fig. 1). Similar in vitro delay in iron incorporation into heme follows from Najean’s experiments.29

After a certain time interval equilibrium between the heme and nonheme pool of radioiron is completed and the value of 60–70 per cent of $^{59}$Fe in the heme does not increase any more. It is necessary to emphasize that 30–40 per cent of radioiron in the nonheme pool after 2–3 hours of incubation of reticulocytes in vitro does probably not reflect the real amount of iron in the nonheme pool of reticulocytes. The same technic was applied to rats. After a 24 hour incorporation we found, on the average, 95 per cent of $^{59}$Fe in heme from the total erythrocyte radioiron.30 Papers of other authors also show a negligible amount of nonheme iron in circulating red blood cells.31,32 It is possible that immature erythroid precursors in the marrow contain a greater amount of nonheme iron but at the stage of reticulocytes the nonheme iron pool is very small.29,32,34 The high percentage of nonheme radioiron is, in our experiments, partly caused by nonphysiologic conditions occurring in in vitro experiments and partly by the deficiency of iron in reticulocytes from the bled rabbit. In both these cases the heme synthesis can be lower. This increases the membrane transport of iron (see below) and permanently maintains a high percentage of radioiron in the nonheme compartment.

The addition of hemin to the incubation medium decreases the incorporation of $^{59}$Fe into heme (Fig. 2). The decrease of specific activity of heme in the presence of hemin can be caused either by the decreased heme synthesis.
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or by an increase in the pool of a nonlabeled intracellular heme which could be brought about by a transfer of heme from medium to reticulocytes. The latter possibility is excluded because it is possible to detect only 12 μg. of hemin originating from medium per 0.2 ml. of reticulocytes if the concentration of hemin in the medium is $3 \times 10^{-4}$M. 0.2 ml. of red blood cells contain about 2 mg. of heme. It means that nonlabeled hemin which either entered the cell or adhered to the surface can decrease the specific activity of heme only to about 0.6 per cent.

The inhibition of heme synthesis by hemin is not surprising and is in agreement with the experiments of Burnham and Lascelles\textsuperscript{36} who were the first to ascertain on bacterial systems that δ-aminolevulinic acid—the first enzyme of the synthetic porphyrin chain—is reversibly inhibited by hemin. Later Karibian and London\textsuperscript{27} using glycine-2-\textsuperscript{14}C proved that the feedback inhibitory effect of hemin on heme synthesis appears also in mammalian reticulocytes. These authors concluded that the feedback control of heme synthesis was at the level of δ-aminolevulinic acid synthetase.

A decreased uptake of radioiron by reticulocytes incubated with hemin could be a consequence of decreased heme synthesis if iron entry into the cell and the rate of heme synthesis are linked. This possibility is also considered by Gallo\textsuperscript{37} who explains decreased $^{59}$Fe incorporation into mice reticulocytes after administration of hemin in vivo as a consequence of decreased heme synthesis. A number of authors\textsuperscript{5,12,13} ascertained that certain concentrations of lead in the incubation medium can inhibit heme synthesis whereby the cellular uptake of iron does not decrease. We confirmed these results in our further experiments, the results of which are summarized in Table 1.

Independence of both processes, i.e., cellular uptake of iron and its incorporation into the heme molecule is supported by a fact well known from clinical hematology. In the so-called siderochrestic anemias, when heme synthesis is inhibited, there is an excess of non heme iron in the marrow erythroblasts and sometimes in the peripheral blood cells.\textsuperscript{38}

On the basis of our experiments with hemin we, therefore, believe that decreased $^{59}$Fe incorporation into reticulocytes is not caused by decreased heme synthesis but by the effect of hemin which prevents the entry of iron into the cell. If this assumption is correct, it does not exclude the possibility that decreased heme synthesis in the presence of hemin may be partly caused by the inhibition of iron transfer across the reticulocyte membrane. This idea is supported by the finding of the parallelism in the inhibition of heme synthesis and iron entry into the cell (Fig. 1, 2) demonstrated by the fact that there is no significant difference in the percentage of total cellular radioiron in heme between control and hemin incubated reticulocytes (Fig. 1). By lowering the amount of intracellular iron, the amount of the substrate necessary for heme synthesis can be reduced and the activity of some enzymes of the synthetic porphyrin chain can be unfavourably influenced. Iron is necessary for the normal activity of δ-aminolevulinic acid synthetase.\textsuperscript{39,40}

The possibility that hemin inhibits heme synthesis in erythroid cells by the
depression of iron entry into these cells is supported by some recent observations. Vavra and Bottomley and Smithee did not obtain evidence for direct inhibitory effect of hemin on the δ-aminolevulinic acid synthetase. We also failed to inhibit the formation of δ-aminolevulinic acid either in hemolysates or in particle preparations from rabbit reticulocytes by hemin used in concentrations in which it reduces heme synthesis in intact reticulocytes. It is noteworthy that also in liver cells δ-aminolevulinic acid synthetase is probably not controlled by end-product inhibition but only by end-product repression by heme.

On the other hand, there are evidences indicating that the heme does not act solely on the reticulocyte membrane. The feedback inhibition of heme synthesis was proved in hemolysates where the insufficient iron supply cannot be taken into consideration. Also the effect of hemin on the formation of δ-aminolevulinic acid is in the meantime not quite excluded for the inhibitory effect of hemin on α-ketoglutaric acid dependent glycine decarboxylation was described. Recently the inhibitory effects of hemin on δ-aminolevulinic acid dehydratase and heme synthetase were reported. Yoshikava did not find, however, the inhibition of heme synthetase by hemin.

The complicated question of the feedback effect of heme on its own synthesis or on iron entry into reticulocytes can be solved only by further experiments.

The results with inhibitors of heme or globin synthesis we consider as further evidences supporting our idea about the regulatory role of heme in the reticulocyte uptake of iron. As inhibitor of heme synthesis we have chosen isonicotinic acid hydrazide (INH) which evokes sideroblastic anemia in man and as well as in animals and serves also as a catalase inhibitor in mycobacteria and the liver. INH is an antagonist of pyridoxin which is essential for glycine and succinyl CoA to form δ-aminolevulinic acid and is also essential for the release of iron from mitochondria of erythroid precursors. INH has probably the most specific influence on heme synthesis from all metabolic inhibitors used up to the present time.

Addition of INH to reticulocytes incubated in vitro inhibits heme synthesis measured by $^{59}$Fe (Fig. 3, Tables 2, 3, 4) and by glycine-2$^{14}$C; the radioactivity of $^{59}$Fe in whole cells, however, increases simultaneously. Even though the incorporation of $^{59}$Fe into reticulocytes with inhibited heme synthesis by INH increases in all experiments, the extent of this increase varies in the different experiments (Table 2). The high statistical significance of increased $^{59}$Fe incorporation into reticulocytes incubated with INH is shown in Table 3.

The variations in the stimulation of $^{59}$Fe uptake by reticulocytes incubated with INH are, according to our preliminary results, caused by the varying saturations of transferrin.

Our experiments, the results of which are summed up in Table 4, excluded the possibility of $^{59}$Fe accumulating on the reticulocyte membrane. In reticulocytes with heme synthesis inhibited by INH the membrane transport of iron must therefore increase. Surplus accumulation of radioiron in nonheme...
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Iron pool under these circumstances can be partially prevented by hemin. (Dotted and interrupted lines, Fig. 1) It is commonly acknowledged that if heme synthesis is inhibited, the iron is accumulated in ferritin and also in other nonheme iron protein. We suppose that also in our experiments with INH, the radioiron accumulates in one or in the other protein or in

It remains to explain decreased incorporation of radioiron into reticulocytes in the presence of cycloheximide which inhibits globin synthesis. The kinetics of incorporation of iron into both reticulocytes and heme after cycloheximide and heme is strikingly similar to the situation appearing during incubation of reticulocytes with hemin (Fig. 4). Grayzel, Fuhr and London have found greater inhibition of globin than heme synthesis after cycloheximide or puromycine and suggested accumulation of free heme in reticulocytes with inhibited globin synthesis. In one of our previous papers we confirmed results of these authors. Moreover, we have found the considerable decrease in the specific activity of heme after purification of hemolysate from cycloheximide treated reticulocytes on the column of Sephadex G-25. These results may be considered as an evidence for the presence of free heme in reticulocytes with inhibited globin synthesis.

It seems probable that accumulation of free heme, when globin synthesis is inhibited, acts as a factor which may be of importance in the decreased entry of iron. Therefore it was interesting to study whether the rate of iron incorporation into reticulocytes incubated with cycloheximide changes if the accumulation of free heme is prevented or diminished by simultaneous inhibition of heme synthesis. INH very probably reduces the amount of free heme when used together with cycloheximide (Table 6) and partly removes the inhibitory effect of cycloheximide on the iron entry into reticulocytes (Fig. 5).

The reduced uptake of iron by reticulocytes probably occurs only if heme is accumulated in sufficient concentration because the iron incorporation is normal in first 15 minutes of incubation with cycloheximide (Fig. 4). The reduction of iron incorporation into reticulocytes is increasing with the growing time of incubation and the difference between incorporation of iron into reticulocytes and heme is diminishing (Table 5).

If cells are preincubated with an inhibitor of proteosynthesis, reduction of iron incorporation into reticulocytes is measurable sooner (Fig. 5). It cannot be excluded that in experiments with INH and cycloheximide the changes in 59Fe incorporation are caused by the direct effect of these substances on the cell membrane. There is, however, much evidence against this possibility. Reduced radioiron uptake by erythroid cells was found after incubation with different inhibitors of globin synthesis. INH increases the incorporation of iron into reticulocytes incubated with cycloheximide to much greater extent than into normal reticulocytes. Hemin is able to inhibit increased Fe incorporation into reticulocytes incubated with INH.

From these results and considerations it follows that in the presence of a surplus of extracellular or intracellular heme, the reticulocyte radioiron uptake...
decreases while, when its formation is inhibited, the iron transfer across the membrane increases. Both these cases prove the common feedback mechanism by means of which the amount of heme can regulate membrane transport of iron. Generally the resulting product controls cellular supply of the principal building material necessary for its synthesis. Heme can either directly influence the membrane receptors for iron-transferrin complex or it can have an inhibitory influence on some unknown transfer system involved in the membrane transport of iron.

The relationship between the iron entry into the erythroid cells and the degree of heme synthesis may be of some importance in the pathogenesis of sideroblastic anemias.

**SUMMARY**

In the presence of hemin $^{59}$Fe incorporation into both heme and reticulocytes decreases. Isonicotinic acid hydrazide which inhibits heme synthesis in vitro is shown to increase reticulocyte radioiron uptake.

During inhibition of globin synthesis by cycloheximide, reticulocyte radioiron uptake decreases. This decrease is probably caused by free heme which accumulates in reticulocytes after inhibition of globin synthesis. Simultaneous addition of an inhibitor of heme synthesis—isonicotinic acid hydrazide—to reticulocytes treated by cycloheximide decreases the inhibitory effect of this antibiotic on the iron entry into reticulocytes.

The authors suppose the feedback linkage by which heme regulates iron entry into reticulocytes.

**SUMMARIO IN INTERLINGUA**

In le presentia de hemina, il occurre un declino in le incorporation de $^{59}$Fe ad in hem e reticulocytos. Hydrazida de acido isonicotinic que inhibi le synthese de hem in vitro se ha provate capace de augmentar Ic acceptation de ferro radioactive per le reticulocytos.

Durante le inhibition del synthese de globina per cycloheximida, le acceptation de ferro radioactive per le reticulocytos declina. Iste declino es probablemente causate per hem libere que se accumula in reticulocytos post le inhibition del synthese de globina. Le addi-
tion simultanee de un inhibitor del synthese de hem—hydrazida de acido isonicotinic—al reticulocytos tractate con cycloheximida reduce le effecto inhibitori de iste antibiotico super le entrata de ferro ad in le reticulocytos.

Le autores postula un nexo retroregulatori per le qual hem regulga le entrata de ferro ad in reticulocytos.

**ACKNOWLEDGMENT**

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