Heme formation in reticulocytes from rabbits and rodents is subject to end product negative feedback regulation: intracellular "free" heme has been shown to control acquisition of transferrin iron for heme synthesis. To identify the site of control of heme biosynthesis in the human erythron, immature erythroid cells were obtained from peripheral blood and aspirated bone marrow. After incubation with human $\delta$Fe transferrin, 2-14Clglycine, or 4-14C$\alpha$-aminolevulinate, isotopic incorporation into extracted heme was determined. Addition of cycloheximide to increase endogenous free heme, reduced incorporation of labeled glycine and iron but not $\delta$-aminolevulinic into cell heme. Incorporation of glycine and iron was also sensitive to inhibition by exogenous hematin ($K_i$, 30 and 45 $\mu$M, respectively) i.e. at concentrations in the range which affect cell-free protein synthesis in reticulocyte lysates. Hematin treatment rapidly diminished incorporation of intracellular $\delta$Fe into heme by human erythroid cells but assimilation of 4-14Cl$\alpha$-aminolevulinic into heme was insensitive to inhibition by hematin ($K_i$ $>$ 100 $\mu$M). In human reticulocytes (unlike those from rabbits), addition of ferric salicylaldehyde isonicotinoylhydrazone, to increase the pre-heme iron pool independently of the transferrin cycle, failed to promote heme synthesis or modify feedback inhibition induced by hematin. In human erythroid cells (but not rabbit reticulocytes) pre-incubation with unlabeled $\delta$-aminolevulinate or protoporphyrin IX greatly stimulated utilization of cell $\delta$Fe for heme synthesis and also attenuated end product inhibition.

In human erythroid cells heme biosynthesis is thus primarily regulated by feedback inhibition at one or more steps which lead to $\delta$-aminolevulinate formation. Hence in man the regulatory process affects generation of the first committed precursor of porphyrin biosynthesis by $\delta$-aminolevulinate synthetase, whereas in the rabbit separate regulatory mechanisms exist which control the incorporation of iron into protoporphyrin IX.

The erythron is the major organ of heme formation in vertebrates. Heme is a component of the blood pigment but also has a regulatory function in erythroid cells, where it coordinates the pathways of hemoglobin biosynthesis (1).

* This work was supported by a grant from The Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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(Received for publication, May 12, 1987)
in the human erythron. Operation of a feedback mechanism affecting delivery of iron from transferrin to erythroid tissues has importance for understanding the genesis of sideroblastic anemia and diseases of iron storage. Likewise, the possibility that infusions of hematin might be used to suppress porphyrin formation in congenital erythropoietic porphyria has been raised by a short-term study (17) but further studies are needed to clarify the site of action of hematin on heme synthesis in human erythrocytes before its general use to inhibit the pathway in man can be accepted.

Accordingly, we have investigated whether feedback control of heme biosynthesis in human reticulocytes and bone marrow tissue occurs, and, if so, how it is regulated. We have also carried out parallel studies using immature erythrocyte cells obtained from the rabbit.

**MATERIALS AND METHODS**

L-Amino acids, δ-aminolevulinate, hemin hydrochloride, cycloheximide, 4,6-dioxohexanoic acid, and rabbit transferrin were obtained from Sigma. Free protoporphyrin IX was supplied by Behring Diagnostics. Ethyl acetate, isonicotinic acid hydrazide, and salicylaldehyde were purchased from Fisher Chemical. Radiochemicals were supplied by Amersham Int. Solutions of heme arginate (Medica Pharmaceutical Co., Finland) used in studies of feedback control were diluted in distilled water and were a generous gift from Dr. R. Oakes, Tillotts Laboratories, United Kingdom. Transferrin was prepared from human serum as described previously (19) and used in the iron-saturation form, unless otherwise stated, at 1 mg/ml of transferrin/ml (25 μM with respect to iron). Other reagents were of analytical grade and from established suppliers.

**Cell Preparations**—Immature erythrocyte cells were freshly obtained from human subjects, seven male and seven female, aged 18-64, with hematologic disorders. Nine subjects had sickle cell anemia; one, recently treated pernicious anemia; one, paroxysmal nocturnal hemoglobinuria; and one, suffered autoimmune hemolyis. The reticulocyte count, as determined by supravital staining with 1% (w/v) new methylene blue, was 10-25% in all subjects studied. Bone marrow aspirates were obtained before, and 1 month after, iron therapy in a man with mild sideropenic anemia due to chronic hemorrhage. Marrow was also obtained from a man with erythroid hyperplasia due to autoimmune hemolyis.

Samples of blood and aspirated marrow were collected into reticulocyte saline buffer (RS): 0.13 M NaCl, 0.0074 M MgCl₂, 0.005 M KCl buffered to pH 7.35 with 0.01 M-Hepes and containing 50 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

**Heme Biosynthesis**—Cells were incubated within 1 h of removal as 10-20% suspensions in RS buffer at 37 °C in the presence of 1 mg/ml iron-saturated transferrin and 0.1% (w/v) D-glucose. The mixture contained, except where stated, 20 L-amino acids at 100 μM each and the incubations were agitated to maintain oxygenation and avoid clumping. In experiments to follow protein synthesis, 35S)methionine (>1000 Ci/mmol) was added at 100-200 μCi/ml and the final concentration of methionine was reduced to 20-50 μM. Overall protein synthesis was determined by counting trichloroacetic acid-insoluble radioactivity incorporated into total cell protein (19), and duplicate determinations were generally within 5% of the mean value.

The ability of heme preparations to stimulate translation of endogenous globin mRNA was checked in rabbit reticulocyte lysates using L-[35S]cysteine as the isotopic label, as reported previously (18). Hemin hydrochloride stock solution was prepared and stored in ethanolic solution as described in Ref. 18 and diluted in H₂O immediately before use; the 40 mM heme arginate stock solution was diluted similarly. Gel filtration experiments using Sephadex G-25 chromatography in RS buffer showed that the two preparations consisted of unpolymerized hematin, which was completely retained by the columns.

**RESULTS AND DISCUSSION**

**Protein Synthesis**— Incorporation of L-[35S]methionine into cell protein by reticulocytes or marrow cells was maintained for at least 90 min during incubation with nutrient medium (Fig. 1). In preparations of rabbit and human reticulocytes, linear incorporation of radiosulfur label was observed, a short-term study (not shown), a lag phase was seen. This lag may be related to the size of the methionine pools
within different cell types. Unlike suspensions of reticulocytes, marrow aspirates contain a large proportion of non-erythroid cells, which might thus explain these differences.

The effects of different concentrations of hematin on cell-free protein synthesis in lysates of rabbit reticulocytes are depicted in Fig. 2. Optimal rates of protein synthesis were peculiar to each lysate and were observed at 10–30 μM hematin. Protein synthesis was rapidly inhibited at higher concentrations. There were no differences in the ability of hemin hydrochloride or heme arginate to restore translational activity in the cell-free system. The concentration of free heme required for maximal protein synthesis in lysates was estimated by adding radiolabeled hematin and separating bound and free ligand by gel filtration. In a lysate with an optimum at 10–15 μM added hematin, the free hematin concentration was less than 2 μM at a final hemoglobin concentration of 150 mg/ml. In intact reticulocytes little or no stimulatory effect of hematin on protein synthesis was seen unless the cells were rendered heme-deficient. This was achieved by incubation with dioxoheptanoate or by removing iron. Maximal rates of protein synthesis were then restored by addition of 20–60 μM hematin (1). These experiments show that the hematin preparations were effective in restoring physiological levels of intracellular heme at the concentrations used.

Heme Synthesis—Rapid biosynthesis of heme by intact immature erythroid cells obtained from humans or rabbits was also sustained in vitro for prolonged periods under the conditions employed (Fig. 3). Heme synthesis in rabbit and human reticulocytes, as judged by following incorporation of 59Fe into cell heme, was linear for 2 h without a lag phase. We attribute the initial lag in the rate of incorporation of glycine to the presence of large pools of unlabeled precursor in the human reticulocyte. Most of the endogenous glycine for δ-aminolevulinate synthesis will be compartmentalized within the mitochondrial matrix and it is likely that equilibration of label with this pool is slow.

Rabbit reticulocytes were more active in heme formation than human reticulocytes, even when samples with similar numbers of reticulocytes were compared. Rabbit reticulocytes exhibited greater dependence on the presence of transferrin iron in the medium for heme synthesis. Maximal rates of incorporation of labeled glycine into heme were observed with 1 mg/ml saturated transferrin. The utilization of radioiron taken up by them for heme synthesis also exceeded the utilization by human erythroid cells. In rabbit reticulocytes from bled, as well as phenylhydrazine-treated animals, utilization was 78 ± 3% (n = 6), whereas in human cells it was 49 ± 2% (n = 7) and 37 ± 8% (n = 3) for reticulocytes and bone marrow suspensions, respectively (p < 0.01). To demonstrate that human transferrin possessed the equivalent capacity to donate iron for heme formation in erythroid cells of both species, rabbit reticulocytes were incubated with iron-labeled human transferrin; the utilization of iron taken up by these cells was 89%. Thus the observed differences in the utilization of iron for heme synthesis by reticulocytes from these species could not be attributed to defective transport activity of the human transferrin preparation.

Feedback Inhibition of Heme Formation in Erythroid Cells—Addition of cycloheximide (40 μg/ml) to suspensions of human erythroid cells in which 59Fe transferrin or 2-[14C]glycine were present inhibited isotopic incorporation into cellular heme (22) (Table I). The effects of cycloheximide might in part be due to reduced synthesis of δ-aminolevulinate synthetase, an enzyme, at least in liver, which has a short half-life. However, studies in rabbit reticulocytes using combined inhibitors of heme and globin synthesis provide evidence that excess heme accumulates in cycloheximide-treated cells and affects iron uptake (11). Addition of exogenous hematin to final concentrations of 20–200 μM also progressively inhibited heme synthesis by the cell suspensions. The concentration of hematin required for 50% inhibition of incorporation of these isotopes into cellular heme (the Kₐ) was found to be 30–45 μM. In rabbit reticulocytes, a similar degree of inhibition of heme synthesis, as determined by the incorporation of 4-[14C]δ-aminolevulinate, was observed; but in human erythroid cells there was a striking lack of sensitivity to exogenous hematin in relation to this precursor (Kₐ 120–>200 μM) (Table I). In human reticulocytes synthesis of porphyrins from 2-[14C]glycine was markedly reduced by addition of hematin, but incorporation of 4-[14C]δ-aminolevulinate into porphyrins was undiminished (Fig. 4).

Effects of Unlabeled Glycine, δ-Aminolevulinate, and Protoporphyrin IX on Heme Synthesis—The kinetics of feedback
Inhibition of heme biosynthesis in immature erythroid cells

Cells from indicated sources were incubated for 90 min with the labeled precursors and either four concentrations of exogenous hematin or 40 μg/ml cycloheximide. Incorporation of substrates was determined after extraction of cellular heme.

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<tr>
<td></td>
<td>K, hematin μM</td>
<td>% inhibition by cycloheximide</td>
<td>K, hematin μM</td>
</tr>
<tr>
<td>Human reticulocytes</td>
<td>30 ± 4 (7)</td>
<td>37 ± 5 (3)</td>
<td>120–&gt; 200 (7)</td>
</tr>
<tr>
<td>Human bone marrow</td>
<td>48 (1)</td>
<td>37 (1)</td>
<td>&gt;200 (1)</td>
</tr>
<tr>
<td>Rabbit reticulocytes</td>
<td>33 ± 4 (9)</td>
<td>31 ± 4 (4)</td>
<td>47 ± 5 (4)</td>
</tr>
</tbody>
</table>

*p < 0.01.

Inhibition of heme biosynthesis in human erythroid cells (depicted in Table I) suggested that formation of porphyrin precursors, rather than availability or chelation of iron was influenced by increased cellular "free" heme. To characterize the regulatory process further, erythroid cells were preincubated with unlabeled precursors which enter the pathway before (glycine) or after (δ-aminolevulinate and protoporphyrin) δ-aminolevulinate synthetase. The results of typical experiments are shown in Fig. 5. Neither glycine, δ-aminolevulinate, nor protoporphyrin affected uptake of radioiron from transferrin. However, synthesis of ^59Fe heme was stimulated by preincubation of reticulocytes and marrow cells with 5 mM δ-aminolevulinate or 25 μM protoporphyrin IX. Preincubation with glycine (5 mM) had some stimulatory action on heme synthesis but the effects of the less diffusible precursor aminolevulinate were greater. Although a greater effect of exogenous δ-aminolevulinate compared with glycine might be explained by differences in pool size, incubation with millimolar concentrations of these heme precursors should have flooded their intracellular pools. Moreover, if the pre-heme pool of glycine were larger, its generation could not be expected to have regulatory significance per se.

The increase in heme synthesis promoted by δ-aminolevulinate and protoporphyrin was also reflected in a striking enhancement of iron utilization within the cell. Treatment of human erythroid cells with δ-aminolevulinate or protoporphyrin overcame the inhibition of heme synthesis induced by low concentrations of exogenous hematin (<40 μM) (Fig. 5) or by cycloheximide (Table II). In human bone marrow, hematin reduced heme synthesis and addition of protoporphyrin and δ-aminolevulinate clearly stimulated both heme synthesis and utilization of intracellular iron, especially at the lower concentrations of hematin.

Addition of hematin progressively reduced the utilization of ^59Fe taken up from transferrin by human erythroid cells, suggesting that heme synthesis was not controlled by the availability of transferrin iron. At higher concentrations of hematin, increased availability of protoporphyrin no longer enhanced the utilization of intracellular ^59Fe for heme synthesis. Thus a further process, which inhibits heme synthesis by reducing utilization of intracellular iron was revealed when flux through the synthetic pathway was stimulated. This process might involve the intracellular delivery of iron to the inner membrane of the mitochondrion. Alternatively, ferrochelatase may itself be subject to direct inhibition by hematin, as has been shown in bovine liver (23).

In rabbit reticulocytes, identical experiments were also carried out to test the effects of unlabeled δ-aminolevulinate and protoporphyrin IX on the uptake and utilization of ^59Fe transferrin for heme synthesis (Fig. 6). These precursors neither affected cellular iron uptake, heme synthesis, nor intracellular iron utilization in immature erythroid cells obtained from the rabbit.

Effects of Increased Cellular Non-heme Iron on Heme Synthesis—Human erythroid cells assimilated less than one-half of the cellular iron into heme (Fig. 5). In human cells, but not the rabbit, however, the utilization of iron was greatly increased when porphyrin precursors of heme were added. To investigate the effects of increasing cellular non-heme iron, ferric salicylaldehyde isonicotinoylhydrazone was added to suspensions of cells. In human erythroid cells, the resting pool of iron in the pre-heme compartment is large and in three separate experiments, treatment with Fe salicylaldehyde isonicotinoylhydrazone neither stimulated heme synthesis from 2-[^14]C]glycine nor modified feedback inhibition induced by hematin (Fig. 7A). In control experiments citrate alone slightly stimulated heme synthesis, as expected, but ferric citrate had no additional effect since it does not donate iron independently of the transferrin cycle (13). In contrast, rabbit reticulocytes obtained from chronically bled or phenylhydrazine-treated animals incorporated into heme more than 75% of the transferrin iron they had taken up. Unlike human reticulocytes, incorporation of 2-[^14]C]glycine into heme was stimulated by Fe salicylaldehyde isonicotinoylhydrazone and...
Regulation of Heme Synthesis in Human Erythroid Cells

FIG. 5. Stimulation of heme synthesis in human erythroid cells. Cell suspensions were preincubated at 4°C with 5 mM glycine (A), 5 mM δ-aminolevulinate (δ-ALA) (Δ), 25 μM protoporphyrin IX (○), or no addition (●), as indicated. The cells were then warmed to 37°C for 90 min in the presence of 59Fe human transferrin in nutrient medium with or without added hematin. Incorporation of 59Fe into whole cells and extracted cellular heme was determined. The extent to which iron taken up by the cells from transferrin was used for heme synthesis is shown as Fractional assimilation. Results are shown for experiments with (A) reticulocytes and (B) bone marrow cells.

the inhibitory action of hematin was attenuated (Fig. 7, B and C). Fe salicylaldehyde isonicotinoylhydrazone stimulated heme synthesis in all four experiments with reticulocytes from bled rabbits and in four experiments using cells from phenylhydrazine-treated animals as previously reported by Ponka and Schulman (14).

Negative feedback control of heme formation was consistently demonstrable in preparations of human erythroid cells incubated under conditions of active protein synthesis. It was associated with a rapid reduction in the absolute fraction of 59Fe taken up from transferrin which could be found in heme. Under control conditions and at the lower concentrations of
Theses) the control rate of incorporation of Fe into heme was with the designated precursors for 30 min at 4 °C and then 90 min at 37 °C in the presence of 1 mg/ml Fe transferrin. Fe was measured in extracted cell heme. In each experiment (number shown in parentheses) the control rate of incorporation of Fe into heme was determined in parallel incubations which contained no addition of protoporphyrin, δ-aminolevulinate (δ-ALA), glycine, or cycloheximide.

<table>
<thead>
<tr>
<th>Condition</th>
<th>% control Fe in heme</th>
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<tr>
<td>Glycine (5 mM)</td>
<td>144 ± 6 (7)</td>
</tr>
<tr>
<td>δ-ALA (5 mM)</td>
<td>177 ± 17 (10)</td>
</tr>
<tr>
<td>Protoporphyrin IX (25 μM)</td>
<td>144 ± 3 (4)</td>
</tr>
<tr>
<td>Cycloheximide (40 μg/ml)</td>
<td>68 ± 7 (7)</td>
</tr>
<tr>
<td>Cycloheximide + glycine</td>
<td>114 ± 10 (4)</td>
</tr>
<tr>
<td>Cycloheximide + δ-ALA</td>
<td>131 ± 10 (7)</td>
</tr>
<tr>
<td>Cycloheximide + protoporphyrin</td>
<td>108 (2)</td>
</tr>
</tbody>
</table>

In human reticulocytes, incubation in iron-free medium had little short-term effect on the rate of incorporation of [14C]glycine into heme, but in rabbit cells, lack of iron reduced heme synthesis profoundly. These data suggest that reserves of non-heme iron exist in human erythroid cells, possibly in ferritin, and that this may be recruited for heme synthesis. It is noteworthy that ferritin is abundant in human erythroid cells and that its concentration falls during erythroid maturation (24). Negative feedback by heme markedly affects iron utilization within the human cell and control of its own synthesis must be exerted either at steps preceding the formation of protoporphyrin IX or at the level of intracellular iron metabolism, including the insertion of ferrous iron into the protoporphyrin nucleus by ferrochelatase.

In rabbit reticulocytes, iron utilization is much greater and heme inhibits the transfer of transferrin iron via the transferrin receptor (10, 11). That the diminished iron uptake induced by low concentrations of hematin in rabbit reticulocytes is matched by a parallel decrease in iron incorporation into heme, further indicates that neither inhibition of protoporphyrin synthesis nor delivery of iron within the cell can alone be rate-limiting for heme formation. Thus in rabbits, iron uptake and intracellular delivery of transferrin iron to ferrochelatase are limiting control steps of heme synthesis.

Apparent differences in the metabolism of iron by human and rabbit erythroid cells might be related to pre-existing iron deficiency in the chronically bled rabbit or by differences in the distribution of cell types in the samples. We believe that the differences cannot be explained by this. The study of heme synthesis in cells obtained from individuals with pre-existing iron deficiency showed that the short-term supply of iron was not rate-limiting. In bone marrow samples, iron uptake is almost entirely restricted to erythroblasts (25) and our observations were remarkably consistent between preparations of human reticulocytes and marrow aspirates. The utilization of iron by human erythroblasts in the present experiments resembles that reported in normal and iron-deficient marrow aspirates by May and associates (26). In previous studies (27) and in the present work, the availability of iron appears to limit the rate of heme synthesis in reticulocytes obtained from animals treated either with phenylhydrazine or by repeated venesection. This is further evidence that pre-existing depletion of body iron stores cannot explain the interspecies differences in heme metabolism identified here.

Addition of protoporphyrin IX in the micromolar concentration range greatly stimulated utilization of iron by human erythroblasts and reticulocytes. Protoporphyrin abolished the effects of low concentrations of exogenous hematin and the endogenous feedback inhibition of heme synthesis induced by cycloheximide treatment. Protoporphyrin had no such action in rabbit reticulocytes. The protoporphyrin effect indicates that in human erythroid cells feedback inhibition of heme formation regulates the biosynthesis of porphyrins rather than the delivery of iron to ferrochelatase or ferrochelatase activity. At high concentrations (>40 μM) exogenous hematin inhibited iron utilization for heme synthesis in human erythroid cells, despite supplementation with protoporphyrin. This indicates that ferrochelatase or intracellular delivery of iron may limit heme formation in human cells when flux through the synthetic pathway is stimulated by relief of a more prox-
inal regulatory step. The failure of Fe-salicylaldehyde isonicotinoylhydrazide to affect heme synthesis further points to ferrochelatase as a secondary control step. In human erythroid cells delivery of iron and activity of ferrochelatase, however, does not, under normal circumstances, appear to limit heme biosynthesis.

In the rabbit reticulocyte, addition of ferric salicylaldehyde isonicotinoylhydrazide enhanced heme synthesis, as determined by incorporation of 2-[14C]glycine (13, 14). The effect of Fe salicylaldehyde isonicotinoylhydrazide was to reduce the inhibitory action of exogenous hemat in heme synthesis. We concur with Ponka and Schulman (12, 14) that in rabbits, acquisition of iron from transferrin regulates heme synthesis by reticulocytes but this view appears to be untenable in relation to the cells of the human erythron.

It has been found that increased free heme inhibits uptake of glycine required for heme biosynthesis in erythroid cells (12). It has also been shown that the plasma membrane of human red cells contains a distinct system for the selective transport of glycine (28). To determine whether δ-aminolevulinate synthesis, and hence porphyrin and heme formation, is subject to control by the availability of intracellular glycine (mediated by the Gly transport system) the effect of preincubating erythroid cells with excess unlabeled glycine was studied. Glycine slightly stimulated heme biosynthesis in human erythroid cells, an effect which probably resulted from mass action on flux through the Shemin reaction. Stimulation of heme biosynthesis in human erythroid cells by δ-aminolevulinate greatly exceeded the glycine effect. Thus formation of this first committed precursor of porphyrin synthesis, rather than the availability of glycine as a substrate, appears to represent the point of metabolic control for biosynthesis of heme.

Erythrocytes and reticulocytes from different species vary in their maturity and in their ability to undertake metabolic reactions (29). However, it is difficult to reconcile inconsistencies in the reported literature (1, 5, 8, 12, 14, 27), particularly those concerning regulation of heme synthesis in rabbit reticulocytes. In several reports the effects of hemin on heme formation at concentrations below 50 μM which are optimal for protein synthesis when added to intact reticulocytes or reticulocyte lysates (18) were slight (1, 5) and their physiological significance is unclear. In the early investigations availability of substrates essential for balanced synthesis of heme and globin was limited and this may account for the dissident conclusions.

Heme regulates its own synthesis in erythroid cells and also stimulates the formation of globin. However, the action of heme on hemoglobin formation differs according to the maturity of the erythroid cell in question. In differentiating erythroblasts and other nucleated cells, heme has pleiotropic effects. In erythroblasts from rabbits, heme greatly promotes transcription of globin mRNA and may also stabilize transcribed globin RNA (30, 31); but in reticulocytes RNA synthesis cannot occur and heme heme acts to sustain translational activity on polyribosomes (2, 3). In the short term, feedback inhibition of heme on its own formation appears to operate by a common mechanism in human reticulocytes and erythroblasts.

This action of heme in the human erythron occurs at a step which precedes formation of the porphyrin macrocycle. In a study of the effects of parenteral hemat in a patient with congenital porphyria, Watson et al. (17) noted a striking reduction in uroporphyrin concentrations in circulating red cells, plasma, and urine shortly after administration of heme. Hitherto it has not been clear as to whether the diminished synthesis of porphyrins was related to an action on nucleated red cell precursors or reticulocytes. We show that addition of exogenous hemat in suspensions of human red cell precursors in vitro directly inhibits the synthesis of porphyrins in both types of cell. Hematin might thus be considered for further evaluation in the treatment of congenital porphyria.

In summary, our findings indicate that heme biosynthesis in human erythroid cells is subject to end product feedback regulation: this occurs at one or more rate-limiting steps which lead to the formation of δ-aminolevulinate. Subsidiary mechanisms appear also to regulate the uptake of iron from transferrin and its incorporation into heme, presumably ensuring that neither excess iron nor unwanted cellular intermediates accumulate.

Acknowledgments—We thank Dr. I. M. London and Prof. L. A. Luzzatto for encouragement and Dra. I. Doka, D. Huang, A. Bashey, and M. Coupe for providing tissue samples. We also thank Jean Black, who kindly typed the manuscript and A. Nunn who generously provided technical assistance during the early stages of this work.

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