The inhibition of δ-aminolevulinic acid (ALA) synthase activity by heme is commonly thought to regulate the overall rate of heme synthesis in erythroid cells. However, since heme inhibits erythroid cell uptake of iron from transferrin, we have tested the hypothesis that in reticulocytes heme regulates its own synthesis by controlling the cellular acquisition of iron from transferrin rather than by controlling the synthesis of ALA. We found that heme added to reticulocytes in vitro inhibits not only the total cell incorporation of $^{59}$Fe from transferrin but also the incorporation of [2-$^{14}$C]-glycine and transferrin-bound $^{59}$Fe into heme. However, hemin did not inhibit [2-$^{14}$C]-glycine incorporation into protoporphyrin. Furthermore, cycloheximide, which increases the level of non-hemoglobin heme in reticulocytes, also inhibited [2-$^{14}$C]-glycine into heme but not into protoporphyrin. With high concentrations of ferric pyridoxal benzoylhydrazone (Fe-PBH), which, independent of transferrin and transferrin receptors, can be used as a source of iron for heme synthesis in reticulocytes, significantly more iron is incorporated into heme than from saturating concentrations of Fe-transferrin. This suggests that some step (or steps) in the pathway of iron from extracellular transferrin to protoporphyrin limits the overall rate of heme synthesis in reticulocytes. In addition, hemin in concentrations that inhibit the utilization of transferrin-bound iron for heme synthesis has no effect on the incorporation of iron from Fe-PBH into heme. Our results indicate that in reticulocytes heme inhibits and controls the utilization of iron from transferrin but has no effect on the enzymes of porphyrin biosynthesis and ferrochelatase. This mode of regulation of heme synthesis may be a specific characteristic of the hemoglobin biosynthetic pathway.

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### MATERIALS AND METHODS

#### Chemicals

Hemin chloride and protoporphyrin were obtained from Porphyrin Products (Logan, Utah), 4,5-dioxoheptanoic acid (succinylacetone) (SA) from US Biochemical Corp (Cleveland) and purified human apo-transferrin from Behringwerke (Marburg, W Germany). All other chemicals used were of the highest purity available. $^{59}$FeCl$_3$ (10 to 25 mCi/mg) and [2-$^{14}$C]-glycine (40 to 60 mCi/mmole) were purchased from New England Nuclear Corp (Boston). Hemin$^*$ was dissolved in 0.15 mol/L NaOH, mixed with an equal volume of 0.15 mol/L Tris-HCl, pH 7.4. The pH of the solution was adjusted to 7.4 with 0.15 mol/L HCl.

#### Rabbit Reticulocytes

Reticulocytosis was induced in male rabbits by repeated bleeding (10 mL/kg/d) by cardiac puncture with the animals under ketan...
ine-HCl (Ketalar, Parke-Davis Canada, Scarborough, Ontario) anesthesia. Blood was defibrinated by shaking it with glass beads for 15 minutes and filtering it through cheesecloth. Cells were collected by centrifugation at 1,650 g for five minutes at 4 °C. The reticulocyte content was enriched by suspending the top quarter of the cell pellet in a washing buffer (5% bovine serum albumin, 0.137 mol/L NaCl/0.005 mol/L KCl/0.001 mol/L Na2HPO4/0.001 mol/L KH2PO4/0.001 mol/L MgSO4/0.1% dextrose, pH 7.0) and centrifuging. This process was repeated three times. Enriched reticulocytes, free of leukocytes, were suspended in incubation mixture containing essential amino acids as defined by Schulman.2

In cytes, free of leukocytes, were suspended in incubation mixture by centrifugation at 1,650 g for five minutes at 4 °C. The reticulo-HCl (Ketalar, Parke-Davis Canada, Scarborough, Ontario) added.

Studies With 59Fe

59Fe bound to either transferrin or pyridoxal benzoylhydrazone.22 PBH was synthesized by Schiff base condensation between pyridoxal and benzoic acid hydrazide.22 PBH was dissolved with a few drops of 1N NaOH and diluted with incubation medium. Following pH adjustment to 7.4, further medium was added to obtain the required PBH concentration. PBH was labeled with 59Fe by the same procedure described previously for the preparation of 59Fe-PBH except that the Fe:PBH ratio was 1:1. After incubation of reticulocytes with 59Fe-labeled ligands, 59Fe was determined in cells that had been washed with PBS and in heme extracted with acid methylethylketone by the method of Teale.23

[2-14C]-Glycine Incorporation Into Heme and Protoporphyrin

Reticulocytes were incubated as described above except that 59Fe-transferrin was replaced by nonradioactive iron-transferrin (20 μmol/L with respect to Fe); 1.5 to 2.0 μCi of [2-14C]-glycine was added per 250-μL sample. At indicated time intervals, cells were washed in PBS, and their content of 14C-heme and 14C-protoporphyrin was determined by means of a procedure modified from Schwartz and Wikoff.24 Samples of packed reticulocytes (25 to 30 μL) were lysed with 500 μL of distilled water and kept frozen overnight. After the samples were thawed, they were mixed with 10 μL of 1 N NaOH containing 10 μg of carrier protoporphyrin and 350 μL of 10% Tween 20. The samples were then mixed with 1 mL of cold ethylacetate-glacial acetic acid (3:1), vigorously shaken, and after settling for 30 minutes at 4 °C, they were centrifuged at 2,800 rpm for five minutes. After the transfer of the upper porphyrin-containing layer into a clean tube, the extraction was repeated and the top layers were combined. After two extractions, the bottom layer was essentially colorless and was discarded. The pooled ethylacetate extracts were washed three times each with 1 mL of 3% sodium acetate. The identities of extracted protoporphyrin and heme were confirmed by paper chromatography26 using commercially obtained heme and protoporphyrin IX as standards. In one experiment, 14C-heme and 14C-protoporphyrin were added separately to reticulocyte lysates, which were then extracted as above. After paper chromatography, 14C-heme and 14C-protoporphyrin were found only in spots corresponding to their respective standards.

The solutions of heme were decolorized in scintillation vials with 300 μL 30% H2O2 and then mixed with 10 mL scintillation cocktail (Biofluor, New England Nuclear). This amount of H2O2 does not decrease the efficiency of counting 14C by more than 5%. The HCl solutions of protoporphyrin were pooled and mixed with 7 mL of saturated sodium acetate. The pH was always above 5.0. Protoporphy was extracted with 2 mL of ethylacetate-glacial acetic acid. 3:1. The extraction was repeated once, and the ethylacetate extracts were pooled and washed twice with 1 mL of 0.1 N HCl to remove coproporphyrin. The protoporphyrin solutions were decolorized and mixed with scintillation cocktail as above.

The identities of extracted protoporphyrin and heme were confirmed by paper chromatography28 using commercially obtained heme and protoporphyrin IX as standards. In one experiment, 14C-heme and 14C-protoporphyrin were added separately to reticulocyte lysates, which were then extracted as above. After paper chromatography, 14C-heme and 14C-protoporphyrin were found only in spots corresponding to their respective standards.

[2-14C]-Glycine Uptake by Reticulocytes

Glycine uptake by reticulocytes was measured essentially as described by Benderoff et al.27 One hundred microliters of reticulocytes was suspended in 700 μL buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl, 12 mmol/L glucose, 1 mmol/L MgCl2, pH 7.4) containing the indicated substances for 30 minutes and then 200 μL [2-14C] glycine (about 2.5 μCi per sample) was added to final concentrations of 0.1 mmol/L or 1 mmol/L. At indicated time intervals, 50-μL samples were withdrawn, mixed with 1 mL ice cold buffer, layered over 150 μL n-butyl-phthalate in Eppendorf tubes and centrifuged at 18,000 g for 15 seconds. 14C was determined both in aliquots of the supernatant buffer and the 5% trichloroacetic acid (TCA) soluble fraction of reticulocyte lysate. The results were calculated for the same volume of intracellular ("In") and extracellular ("Out") water. Intracellular water was assumed to be 70% of the packed volume of reticulocytes as determined by hematocrit.27

RESULTS

Effects of Succinylacetone (SA) and 2,2'-Bipyridine on [2-14C]-Glycine Incorporation Into Heme and Protoporphyrin

Because the major goal of this study was to elucidate the step (or steps) at which hemin controls heme synthesis, the effects of two well-defined inhibitors of heme synthesis on the incorporation of [2-14C]-glycine into heme were measured first.

Figure 1 shows that 1 mmol/L of SA inhibits [2-14C]-glycine incorporation into both heme and protoporphyrin. This is not unexpected because SA inhibits ALA dehydratase28,29 and therefore inhibits the synthesis of proporphobilinogen. On the other hand, 2,2'-bipyridine inhibits [2-14C]-glycine incorporation into
heme, but stimulates its incorporation into protoporphyrin, since the last step in heme biosynthesis is inhibited because of the binding of Fe$^{2+}$ by 2,2'-bipyridine.\textsuperscript{15} Contrary to the effect of SA with 2,2'-bipyridine, the synthesis of heme and protoporphyrin when taken together is not decreased below control values.

It was important for us to determine that the protoporphyrin we extract from the cells is derived from a pool that has the kinetic properties of a precursor of heme. Reticulocytes were incubated with [2-\textsuperscript{14}C]-glycine for 30 minutes, washed, and reincubated with or without 2,2'-bipyridine or SA as described in Fig 2. In control cells, there is a continuous increase in \textsuperscript{14}C-heme during the next 60 minutes of incubation, but only about 20% of this increase is at the expense of \textsuperscript{14}C-protoporphyrin. This is probably because of the intracellular content of \textsuperscript{14}C-labeled precursors of protoporphyrin including [2-\textsuperscript{14}C]-glycine. The incorporation of \textsuperscript{14}C into heme is inhibited by 2,2'-bipyridine by about 55% and causes an accumulation of \textsuperscript{14}C-protoporphyrin, whereas SA inhibits the incorporation of \textsuperscript{14}C into heme by about 40%; almost all of the \textsuperscript{14}C that does appear in heme can be accounted for by the decrease in \textsuperscript{14}C-protoporphyrin, however. In 15 experiments, the ratios of \textsuperscript{14}C-heme to \textsuperscript{14}C-protoporphyrin varied between 0.15 and 2.28 after 60 minutes of incubation with [2-\textsuperscript{14}C]-glycine. Although difficult to explain with certainty, this might reflect variations in ferrochelatase levels or activities in reticulocytes of different maturity.

**Effect of Heme on Heme Synthesis**

Figure 3 shows the results of a typical experiment and Table 1 summarizes the results of several experiments in which reticulocytes were incubated with and without hemin in the presence of either \textsuperscript{59}Fe-transferrin or [2-\textsuperscript{14}C]-glycine. Hemin, in a concentration-dependent manner, inhibited \textsuperscript{59}Fe incorporation into whole cells and heme and [2-\textsuperscript{14}C]-glycine incorporation into heme. However, at 60 minutes 10 \textmu mol/L and 25 \textmu mol/L hemin stimulated [2-\textsuperscript{14}C]-glycine incorporation into protoporphyrin by 13% and 17%, respectively, and 50 \textmu mol/L hemin, which inhibited \textsuperscript{59}Fe incorporation into heme by 80%, inhibited [2-\textsuperscript{14}C]-glycine incorporation into protoporphyrin by only 8% (Table 1).

Hemin can be taken up by erythroid cells, incorporated into hemoglobin,\textsuperscript{4,10} and can undoubtedly also...
increase the size of the intracellular pool of non-hemoglobin heme. The level of intracellular non-hemoglobin heme can also be increased in reticulocytes by inhibiting globin synthesis. Figure 4 shows that cycloheximide inhibited [\(2-{^14}\text{C}\)]-glycine incorporation into heme by 33%, but had no effect on [\(2-{^14}\text{C}\)]-glycine incorporation into protoporphyrin.

**Effect of Heme on Uptake of Glycine by Reticulocytes**

Because a change in the uptake of a labeled substrate would alter the rate of its appearance in products of its metabolism, we examined the effect of heme on glycine uptake by reticulocytes. Figure 5 shows that hemin inhibited the transport of [\(2-{^14}\text{C}\)]-glycine added to final concentrations of 0.1 mmol/L and 1.0 mmol/L into both control and SA-treated reticulocytes. At a concentration of 0.1 mmol/L of [\(2-{^14}\text{C}\)]-glycine, the inhibition of the glycine uptake was found with a concentration of hemin as low as 10 \(\mu\text{mol/L}\).

**Effect of Heme on Uptake and Utilization of Iron From Fe-PBH**

Although transferrin is considered an obligatory donor of iron for erythroid cells, we demonstrated previously that Fe-PIH, independent of transferrin and transferrin receptors, can provide iron for heme synthesis in reticulocytes. More recently, we found that Fe-PBH, an analogue of Fe-PIH, is more effective than Fe-PIH as an iron donor. (P.P., H.M.S., unpublished observations, 1984). The uptake of iron from transferrin by reticulocytes in vitro is saturable with respect to Fe-transferrin concentrations, maximum rates being obtained within the physiologic range of plasma iron concentration (20 \(\mu\text{mol/L}\)). At physiologic concentrations of iron, the uptake of iron from Fe-PIH and Fe-PBH is less than from transferrin; however, the rate of iron uptake from the synthetic ligands is not saturable in concentrations from 20 to 200 \(\mu\text{mol/L}\). (P.P., H.M.S., unpublished observations, 1984).

With 200 \(\mu\text{mol/L}\) of Fe-PBH, the uptake of iron and its incorporation into heme is much greater than with the saturating concentration (20 \(\mu\text{mol/L}\)) of Fe-transferrin (Table 2). It seems that the amount of heme synthesized by the cells can be increased by supplying
iron to the cells in a form that does not depend on the transferrin pathway of iron utilization. This could mean that the rate of iron utilization for heme synthesis in reticulocytes is not limited by either the rate of protoporphyrin synthesis or the activity of ferrochelatase. It suggests that the number of transferrin receptors, the processing of transferrin-receptor complexes, the rate of release of iron from transferrin, or the movement of iron from transferrin to ferrochelatase may be the limiting factor for heme synthesis. Therefore, since hemin inhibits the utilization of iron from transferrin, it was of interest to see if hemin affected the utilization of iron from Fe-PBH. Figure 6 shows that 50 μmol/L hemin had virtually no effect on the incorporation of iron from Fe-PBH into heme, whereas a similar concentration of hemin inhibited the incorporation of iron from transferrin into heme by more than 75%.

These results suggest that under normal conditions, the iron for heme synthesis in reticulocytes is derived directly from transferrin and that intracellular iron stored in ferritin and other compounds is not available for this purpose. This idea is in accord with results published recently by us.29

DISCUSSION

It is generally believed that end product feedback inhibition regulates heme biosynthesis.2,3,32,34 Burnham and Lascelles first demonstrated that hemin inhibits both the activity and synthesis of ALA synthase in *Rhodopseudomonas spheroides*.35 Karibian and London later showed that heme synthesis in erythroid cells is subject to negative feedback control, and they suggested that heme regulates ALA synthase activity in these cells also. Other studies, however, have shown that heme inhibits and controls iron uptake from transferrin in erythroid cells.4 In reticulocytes, heme does not inhibit the binding of transferrin to receptors but seems to interfere with the release of iron from transferrin, suggesting that the overall rate of heme synthesis in erythroid cells may be regulated by feedback inhibition of the uptake of iron from transferrin.

There is considerable experimental evidence for this hypothesis. First, the rate of iron uptake from transferrin limits the rate of heme synthesis in reticulocytes. This is supported by the following observations: (1) lack of Fe-transferrin reduces the utilization of glycine for heme synthesis;15 (2) elevation of the non-heme iron pools in reticulocytes increases the incorporation of glycine into heme3; (3) addition of ALA or protoporphyrin to reticulocytes does not increase 59Fe incorporation into heme16,29; and (4) the increased uptake of iron from Fe-PBH as compared to transferrin is accompanied by an increase in 59Fe (Table 2) incorporation into heme. Second, heme has no effect on the utilization of intracellular non-heme 59Fe for heme synthesis,14,15 but does inhibit the incorporation of transferrin-bound 59Fe into heme (Figs 3 and 6) and this inhibition is not reversed by heme.16,18 Third, heme not only inhibits glycine but also ALA incorporation into heme.16,18 Fourth, hemin, in concentrations in which it inhibits heme synthesis in intact cells21 has no effect on the synthesis of ALA by mitochondria isolated from reticulocytes14 or erythroid fetal liver16 or by homogenates of mouse spleen erythroid cells.37 There are reports that heme inhibits ALA synthase purified from erythroid cells;38,39 however, the concentration of hemin used was so high that the activities of other enzymes, such as succinate dehydrogenase, isocitrate dehydrogenase, and NADH-cytochrome c reductase would also be inhibited.30

Even though, when taken together, these results strongly suggest that ALA synthase is not the rate-limiting enzyme and site of regulation of heme synthesis in reticulocytes, this view has been questioned.18,41 Ibrahim et al concluded that hemin inhibits ALA
synthase activity in reticulocytes; however, more recently he and his co-workers demonstrated that hemin induces ALA synthase activity in erythroid precursor cells during differentiation. It is difficult to imagine opposite responses of ALA synthase to heme in erythroid precursor cells and reticulocytes. In their experiments with reticulocytes, using transferrin-free medium, they found that 50 to 100 μmol/L hemin inhibited [2-14C]-glycine incorporation more than [4-13C]-ALA incorporation into heme. However, their conclusion that heme controls ALA synthase activity in reticulocytes may not be justified. The results of studies of heme synthesis in reticulocytes incubated without transferrin as a source of iron are questionable. In addition, because hemin inhibits glycine transport (Fig 5), their measurements of the effect of hemin on the rate of heme synthesis using [2-14C]-glycine incorporation were probably incorrect. Our experiments with 1.0 and 0.1 mmol/L glycine show that hemin inhibits glycine uptake more at the lower concentration of glycine (Fig 5). The inhibition of transport could have been quite considerable in their experiments, for they used only 12.5 μmol/L glycine. Furthermore, such a low concentration of glycine may not reflect ALA synthase activity accurately because the K_m for glycine of the enzyme is 4 to 19 mmol/L. Because of these difficulties of interpretation, we decided to test further the hypothesis that heme regulates heme synthesis in erythroid cells by controlling the entry of iron into the cells and/or the delivery of iron to ferrochelatase.

When hemin was added to reticulocytes in relatively low concentrations (10 to 25 μmol/L), it inhibited 59Fe and [2-14C]-glycine incorporation into heme but stimulated [2-14C]-glycine incorporation into protoporphyrin (Fig 3). By correcting for the decreased uptake of [2-14C]-glycine caused by hemin (Fig 5), we were able to demonstrate that even 50 μmol/L hemin leads to accumulation of 14C-protoporphyrin. Although 50 μmol/L hemin inhibits glycine uptake by the cells, it does not significantly affect its incorporation into protoporphyrin. It is apparent that the level of 14C-protoporphyrin in the cells is determined not only by the flux of [2-14C]-glycine into the cells but also by the rate of 14C-protoporphyrin incorporation into heme. We believe that these results clearly show that hemin does not inhibit any enzyme in the heme biosynthetic pathway between ALA synthase and protoporphyrinogen oxidase, but does inhibit heme synthesis by blocking the uptake of iron from transferrin. Even though heme has been shown to inhibit the activity of ferrochelatase from liver mitochondria, our results indicate that it does not inhibit ferrochelatase in reticulocytes since hemin has no effect on the incorporation of iron from Fe-PBH into heme.

The mechanism by which heme regulates heme synthesis may be different in erythroid and nonerythroid cells, a conclusion that is in agreement with a review by Kappas et al. The inhibition of iron uptake that is caused by heme seems to be a property only of erythroid cells since it is observed with reticulocytes (references 4 through 9 and this study), fetal mouse liver erythroblasts, human erythroblasts, and hemoglobin-synthesizing Friend erythroblasts. The inhibition of iron uptake from transferrin, hemin, and transferrin-free conditions is not with uninduced Friend erythroblasts, transferrin, and rat hepatocytes (P.P., H.M.S., unpublished observations, 1983). Recent results from our laboratory suggest that in addition to its effect on iron uptake from transferrin, heme may also regulate the expression of transferrin receptors in erythroid cells. It is possible that the control of heme synthesis may involve ALA synthase only in nonerythroid cells, since different isoenzymes are found in erythroid and hepatic cells. The activities of partially purified cytosolic and mitochondrial ALA synthase from liver are inhibited by relatively low concentrations of heme. The transcriptional control of ALA synthase may also be different in erythroid and nonerythroid cells because hemin (0.1 μmol/L) inhibits the synthesis of ALA synthase in hepatocytes but does not repress the synthesis of the enzyme in erythroid cells. On the contrary, heme can be an inducer of ALA synthase in erythroid cells.

In conclusion, in erythroid cells, heme neither inhibits nor represses enzymes of porphyrin biosynthesis. However, the level of "free" intracellular heme, which is determined by the rate of globin synthesis, limits heme synthesis by regulating the availability of iron for ferrochelatase activity. Because some step in the movement of iron from extracellular transferrin to ferrochelatase is both limiting and controlled in erythroid cells, it may be a specific part of the hemoglobin biosynthetic pathway, as was suggested recently by us.

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