Characterization and Measurement of Heme Synthetase in Normal Human Bone Marrow

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The insertion of iron into protoporphyrin is the final step of a series of reactions in the biosynthesis of heme. Several previous investigations have demonstrated that this step is enzyme-dependent and the enzyme has been named "heme synthetase" or "iron chelatase." The enzyme has been extensively characterized in rat liver mitochondria, and Porra and Jones suggested that two enzymes may be involved in the reaction.

The enzymic biosynthesis of heme from iron and protoporphyrin in erythroid tissue has mainly been described in avian erythrocyte lysates. Langelaan, Losowsky and Toothill have recently demonstrated heme synthetase activity and some properties of the enzyme in normal human peripheral blood. Decreased heme synthetase activity in peripheral blood has been reported in one patient with pyridoxine-responsive anemia and also in several types of hematological disorders. Lochhead and Goldberg measured heme synthetase in water lysates of human bone marrow and Steiner has described low heme synthetase activity in water lysates of bone marrow from patients with thalassemia.

In the present studies, a method for assay of heme synthetase in human bone marrow was developed. Employing this procedure, the enzyme was characterized. Values for activity of the enzyme in normal marrow were established and, in part, confirm the work of Steiner. In addition, some factors affecting the activity of the enzyme are described. A preliminary report of these findings has been made.

Materials and Methods

Bone marrow was obtained from the posterior iliac crest of healthy volunteers ranging in age from 22 to 50. Using the Vim-Silverman needle, approximately 10 ml. of a marrow suspension was usually obtained from one site. Frequently up to 30 ml. of adequately cellular material was aspirated. Sodium EDTA (10 mgm./ml.) was used as anticoagulant, all glassware and pipettes were iron-free, and all manipulations of the marrow tissue until incubation for enzyme assay were carried out at 4 C.
**Preparation of Marrow Lysate**

Marrow particles were dispersed by repeated aspiration through a 20-gauge needle. The adequacy of the specimen was determined by a total nucleated cell count which varied from 20,000 to 50,000/mm.³. A specimen with a count less than 20,000/mm.³ was discarded. The cells were then washed three times with 0.85 percent NaCl and suspended in 2 volumes of Krebs-Ringer-Phosphate buffer, pH 7.4. From this suspension, the final nucleated cell count and differential were determined and then a hemolysate prepared by quick-freezing and thawing. Microscopical examination of the resulting hemolysate showed complete rupture of all cell membranes.

**Enzyme Assay**

The assay of heme synthetase activity consisted of incubation of 4.5 ml. hemolysate with 0.5 ml. protoporphyrin solution (containing 112.5 mcgm. to give a final concentration of 3.6 × 10⁻⁵M) and 0.5 ml. ferrous iron solution (containing 5.8 mcgm. with a specific activity of 1.6 mc./mgm. to give a final concentration of 1.9 × 10⁻⁵M) for 30 minutes in a shaking water bath at 37 C. in room air. The reaction was stopped by stirring the flask contents into a 1:4 solution of 2 percent strontium chloride in glacial acetic acid and acetone. Heme was isolated by the method of Labbe and Nishida¹³ with a recovery of 60 percent. The specific activity of the isolated heme was determined in a Baird Atomic sample changer (Model 707) with a counting efficiency of 30 percent. The absolute counts per sample were obtained by correcting for a 100 percent yield of heme as calculated from the hemoglobin concentration of the hemolysate. Enzyme activity was expressed in millimicromoles of heme formed per one million nucleated erythroid precursors. With duplicate or triplicate determinations of enzyme activity, the average variation was ±4 percent with a range of 1.1 percent to 6.3 percent.

To evaluate variation in the percent of radioiron incorporation which might be produced by differences in the size of the iron pool of different individuals, a sample of bone marrow lysate was analyzed for total iron according to a modification of the method of Lorber.¹⁴

Cell counts and hemoglobin determinations were performed using standard techniques.¹⁵

Protoporphyrin was prepared as the methyl ester by the method of Crinstein¹⁶ and its purity determined by melting point determination and spectral characteristics using a Cary recording spectrophotometer. Free protoporphyrin was obtained by hydrolysis of the methyl ester with 3N HCl and preserved over NaOH in a dessicator. For enzyme assays, a fresh solution of protoporphyrin was prepared by dissolving it in 0.05 percent NaOH. Undissolved protoporphyrin was removed by filtration and the pH of the filtrate adjusted to pH 7.8 with 0.5 percent HCl. To prepare the proper concentration of protoporphyrin accurately, the freshly prepared solution was analyzed fluorometrically and diluted appropriately.

Ferrous-⁵⁹ citrate was obtained from Squibb and its specific activity varied from 6.8-29.3 mc./mgm. of iron. The desired iron concentration was obtained by diluting the ferrous-⁵⁹ citrate with a ferrous chloride solution.

Heme was obtained commercially as the hemin chloride.

**RESULTS**

**Characterization of the Heme Synthetase Reaction**

**Linearity with time of incubation.** The rate of heme synthesis was determined at varying times of incubation up to 60 minutes. As seen in Figure 1, the reaction rate was found linear with time during the initial 30 minutes but began to drop off at 45 minutes.

**Effect of pH.** Enzyme activity was studied over a pH range of 6.9-8.4 employing phosphate buffer. The activity was maximal at pH of 7.4 (Figure 2).

**Michaelis constants.** The Km was determined for both substrates by the method of Lineweaver and Burke¹⁷ using substrate concentrations from 0.86 × 10⁻⁵M to 5.08 × 10⁻⁵M for iron and from 1.0 × 10⁻⁵M to 9.3 × 10⁻⁶M.
for protoporphyrin. Plots of the initial velocities gave Km values of 1.8 for $10^{-6}$M for protoporphyrin and $1.7 \times 10^{-5}$M for iron (Fig. 3).

*Effect of enzyme concentration.* At optimal substrate concentrations, the effect of 1:1.5 and 1:2 dilutions of the lysate on the rate of heme synthesis was determined. Enzyme activity was proportional to enzyme concentration over the range studied (Fig. 4).

*Stability of heme synthetase.* Heating the hemolysate at 56 C. for 30 minutes destroyed 96 percent of enzyme activity.

**Heme Synthetase Activity in Normal Human Bone Marrow**

The activity of heme synthetase in marrow from 17 healthy volunteers is depicted in Figure 5. It ranged from 0.056–0.150 millimicromoles of heme/
Other Studies of Heme Synthetase

The effect of several substances on heme synthetase activity was studied and the results are presented in Table 1. Ascorbic acid at $1 \times 10^{-3}M$ concentration increased enzyme activity to 176 percent of control but higher concentrations were inhibitory. Glutathione enhanced enzyme activity 54 percent at
1 × 10⁻²M concentration but was inhibitory at 4 × 10⁻³M. Pyridoxal-phosphate at 1 × 10⁻³M concentration inhibited enzyme activity to 54 percent of control.

The effects of high concentrations of the substrates of the reaction on heme synthetase activity were also studied. At four times the optimal concentration of protoporphyrin, there was no inhibition of activity but at thirteen-fold concentration, inhibition was appreciable and progressively increased with higher concentrations. A seven times optimal concentration of ferric or ferrous
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iron did not inhibit the enzyme. When iron bound to transferrin was included in the assay, it was not utilized for heme synthesis.

The effect of hemin on heme synthetase was studied at concentrations ranging from $0.5 - 10^{-4}$M to $4 \times 10^{-4}$M. Figure 6 shows the effect of concentration of added hemin on enzyme activity. Inhibition was appreciable at $0.5 \times 10^{-4}$M and was progressively increased at higher concentrations.

**Discussion**

It is well established that heme synthetase in liver tissue is located in the mitochondrial fraction of the cell. Since only immature erythroid cells, including the reticulocytes, synthesize hemoglobin, it has been inferred that heme synthetase activity is dependent upon the presence of mitochondria in erythroid tissue. Schwartz et al. have demonstrated that only the particulate fraction of erythroid cells possesses heme synthetase activity. The avian erythrocyte enzyme was reported to be unstable on storage. The observations presented here indicate that any structural integrity of mitochondria which might be destroyed by quick freezing and thawing is not necessary for measurement of activity of the human enzyme.

The studies described above indicate that the formation of heme from iron and protoporphyrin in human red cell precursors is an enzyme-dependent step. The enzyme is inactivated by heat and has a pH optimum. The reaction rate is linear with time and is proportional to enzyme concentration. The optimal pH for the human heme synthetase is the same as that in rabbit bone marrow but differs from the avian erythrocyte enzyme (pH 7.9–8.0) and rat liver enzyme (pH 8.2). Langelaan et al. reported that the optimal pH of the enzyme in peripheral blood is 8.2 and presumably, in this case, the reticulocyte enzyme was measured. The optimal concentration of protoporphyrin found for human marrow heme synthetase is very similar to that previously described in the avian species as well as for the liver enzyme. Detailed data of optimal iron concentration have not been reported previously.

The normal values for heme synthetase activity in human bone marrow obtained by the procedure reported here (0.053 percent to 0.141 percent of iron-59 incorporation/10⁶ nucleated red cells) compare with those reported by Steiner. In a water hemolysate preparation, he found a range of 0.067 percent to 0.077 percent iron-59 incorporation per $1 \times 10^6$ erythroid cells in 6 normal individuals. It has been suggested that measurement of heme synthesis with radioiron as a tracer may produce error when there are variations in the size of the iron pool. In the assay system described, iron is added in amounts approaching the optimal substrate concentration rather than in trace quantities as used in most previous studies. Therefore, it would be expected that dilution by free endogenous iron present, if any, in the marrow cell lysate would be minimized. Since the total iron in the hemolysate preparation which was assayed for enzyme activity varied little from hemoglobin iron, the added radioiron would not have been significantly diluted by non-heme iron in normal marrow. Whether any endogenous iron is in such a form that it would freely exchange with the added iron is not known. To what extent excess non-heme
iron present in disease states might affect values in the assay is under study.

A stimulatory effect on the enzyme was observed when ascorbic acid was added to the incubation mixture. This finding is in agreement with previous observations in avian and rabbit hemolysates. Precisely how ascorbic acid increases the enzyme activity remains to be determined with an assay system using a purer enzyme preparation. Since it is a reducing substance, it may stabilize the enzyme by activation of its sulfhydryl groups; it may influence the release of iron from a bound form or it may maintain iron in the ferrous state.

A concentration of $1 \times 10^{-2}$M of glutathione was necessary to enhance enzyme activity. Schwartz et al. have delineated glutathione as a dialyzable substance which restored enzyme activity to avian hemolysates after their inactivation by dialysis. Others have found an enhancement of avian heme synthetase activity by glutathione. Presumably, this substance also stimulates by activation of sulfhydryl groups. Pyridoxal phosphate at $1 \times 10^{-6}$M concentration inhibited enzyme activity. The mechanism of this inhibition is not clear but pyridoxine may chelate iron and thus decrease the availability of this substrate.

Some inhibition of heme synthetase by protoporphyrin has been demonstrated in avian red cell lysates. The protoporphyrin concentration producing this inhibition as well as the inhibition demonstrated here is considerably above the normal cellular concentration of free protoporphyrin. In erythropoietic protoporphyria, protoporphyrin may reach levels of several thousand micrograms percent. In this disorder, red cell and hemoglobin production appear to be normal. The inhibitory effect of protoporphyrin may only be of interest as an in vitro finding and would not appear to be a negative controlling factor of enzyme activity in vivo.

Five-fold concentrations of optimal ferric or ferrous iron do not inhibit the enzyme. This is contrary to the studies in rabbit marrow but in agreement with the recent study of Bishop and Hathaway that iron does not inhibit heme synthesis. The finding that iron bound to transferrin was not utilized in the present system indicates that hemolysis appears to destroy the mechanism which specifically and actively removes iron from transferrin for incorporation into heme.

It is well accepted that the rate of heme synthesis governs the rate of hemoglobin synthesis and red blood cell production. For example, in iron deficiency, heme synthesis is impaired because of lack of iron, and in chronic infection, at least in part, because of impaired transport of iron to the erythroid precursors. In the past few years, several investigations at the subcellular level have indicated that the rate of heme synthesis may be controlled by several feedback mechanisms within the biosynthetic pathway of heme. Karibian and London have indirectly shown in rabbit reticulocytes that hemin inhibits the first step in heme synthesis, namely, the formation of delta-aminolevulinic acid. Direct inhibition of delta-aminolevulinic acid synthetase from rabbit and duck reticulocytes by hemin has also been observed. A feedback inhibition by heme of porphyrin synthesis at the level of human erythrocyte delta-
Heme synthetase in normal human bone marrow

Aminolevulinate dehydrase has been observed. Gallo has recently demonstrated an inhibitory effect of hemin on heme formation from C-14-glycine and from iron-59, in vivo. The observations reported here indicate that this inhibition may occur through an effect of heme on the heme synthetase step.

In addition, the rate of heme synthesis appears to be closely linked to globin synthesis. Globin stimulates the enzymic synthesis of heme from iron and protoporphyrin in an avian erythrocyte lysate fraction. Also, heme increases globin synthesis and coordinates alpha and beta chains in this process in rabbit reticulocytes and augments the release of the completed hemoglobin molecule from ribosomes.

Whether free heme is found in the cell in normal or pathological states has not been determined. If free heme is a normal intracellular substance, the inhibition of heme synthetase by its product heme would represent another regulatory mechanism of heme biosynthesis.

**Summary**

The heme synthetase step in human bone marrow has been characterized in a crude lysate as an enzymatic reaction in that it has a pH optimum of 7.4, is heat labile, and at optimum substrate concentrations is linear with time and enzyme concentration over the first 30 minutes. The Km of iron was found to be $1.7 	imes 10^{-9}$ M and for protoporphyrin $1.8 	imes 10^{-6}$ M.

High iron concentrations do not affect the enzyme but ascorbic acid and glutathione were found to augment the activity of the enzyme. Pyridoxine and high protoporphyrin concentrations inhibit heme synthetase activity. A negative feedback control of heme synthetase by heme is present in human red cell precursors.

**SUMMARIO IN INTERLINGUA**

Le passo synthetase de heme in human medulla ossee esseva characterisate in un lysato crude como reaction enzymatic in tanto que su optime pH es 7.4, que illo es thermolabile, e que—a optime concentrationes del substrato—illo es relationate linearmente con le tempore e con le concentration del enzyma durante le prime 30 minutas. Esseva trovate que le Km de ferro es $1.7 	imes 10^{-9}$ M; pro protoporphyrina illo es $1.8 	imes 10^{-6}$ M.

Alte concentrationes de ferro non affice le enzyma, sed il esseva constatate que acido ascorbic e glutathiona augmenta le activitate del enzyma. Pyridoxina e alte concentrationes de protoporphyrina inhibi le activitate de synthetase de heme. Un negative regulation reactive del synthetase de heme per heme es presente in human precursores de erythrocyto.

**REFERENCES**

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