

THE BIOSYNTHESIS OF HEMOGLOBIN FROM IRON, PROTOPORPHYRIN AND GLOBIN * †

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The formation of heme in a system containing protoporphyrin, iron and a soluble preparation from avian erythrocytes has been studied by several investigators (3-6). The observations that the biosynthetic reaction is inactivated by heating at 56° C for 30 minutes, has a pH of optimal activity and stability near neutrality with a lower rate on each side of this optimum, and that the rate of heme synthesis is proportional to enzyme concentration, suggest, although admittedly do not prove, that the reaction is enzyme-dependent (4-6). The enzyme has been tentatively named "heme synthetase" (2, 5). However, since more than one enzyme may be involved in this reaction, the name "heme synthetase system" would be more appropriate until it can be shown that a single enzyme is involved.

The activity of the heme synthetase system is increased by reduced glutathione, cysteine, or other reducing agents (4-7). Inhibition with *p*-chloromercuriphenylsulfonate and with iodoacetamide suggests that active sulfhydryl groups are required for enzyme activity (5).

Purification of the heme synthetase system and augmentation of heme synthesis by globin are described in the present studies. Although several

investigators have studied the synthesis of heme and globin in whole cell systems (8, 9), the effect of globin on the enzymic synthesis of heme in a purified system has not been demonstrated previously.

METHODS

Assay procedure

Reaction mixtures contained 6 ml of enzyme preparation, 0.5 μ mole protoporphyrin, 0.13 μ c ferrous (Fe^{59}) citrate, 10 μ moles cysteine, and 1,000 μ moles Tris [tris (hydroxymethyl) aminomethane] buffer, pH 7.8, in a total volume of 10 ml. The specific activity of the Fe^{59} ranged from 1 to 3 mc per mg of iron. The reaction mixtures were incubated with shaking at 37° C for 30 minutes. The reaction was then stopped by adding 25 ml of a "carrier" hemoglobin solution which contained 1×10^{-2} M potassium cyanide. The heme was crystallized twice and the radioactivity was determined (3, 5). Heme synthesis was expressed as the per cent incorporation of added radioiron into heme (per cent uptake of Fe^{59}) or as the total radioactivity in the heme (cpm). A single pool of chicken blood was used for a given experiment. In this way variations in iron uptake due to differences in the size of the iron pool from one sample of blood to another were eliminated.

Source of materials

Washed chicken erythrocytes, prepared as described previously (3, 5), were used as a source of enzyme. Purified albumin and γ -globulin were obtained from human plasma by preparative curtain electrophoresis (10). The transferrin used was Cohn fraction IV-7 (11). Globin was prepared by acid acetone precipitation (12). Saponin (Eastman Kodak Company) and Tween-20 (Atlas Powder Company) were obtained from commercial sources.

Preparative procedures

Starch block electrophoresis was carried out as described by Kunkel, Cepellini, Müller-Eberhard and Wolf (13). Column chromatography was performed as described by Allen, Schroeder and Balog (14). Except where stated, all of the preparative procedures were performed at 4° C.

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Analytical procedures

Hemoglobin concentrations were determined by the cyanmethemoglobin method in a Beckman model DU spectrophotometer (15). Nitrogen analyses were performed by a micro-Kjeldahl method (16).

Preparation of enzyme solutions

Step 1: preparation of hemolysate. Forty ml of washed, packed erythrocytes was hemolyzed (17) with 60 ml of 0.15 M KCl containing sufficient saponin to give a final saponin concentration of 0.03 per cent. This concentration of saponin was found to give maximal hemolysis with minimal enzyme inactivation. The hemolysate (fraction I) was incubated at 5° C for 30 minutes with intermittent mixing. One hundred ml of cold 0.15 M KCl was added and the hemolysate was centrifuged at 1,300 G for 60 minutes. The supernatant solution (fraction II), which was inactive, was removed and saved for the recombination experiments.

Step 2: removal of hemoglobin. The residue was washed with 100 ml of chilled 0.15 M KCl and centrifuged at 1,300 G for 60 minutes. The supernatant solution was removed by suction. The residue was transferred to a clean glass centrifuge bottle. In this manner the residue could be separated from the few unhemolyzed red cells which adhered to the glass surface. The residue was washed with 0.15 M KCl (10 to 15 times) until it appeared light yellow in color and was visually free of hemoglobin. On examination with a phase microscope, this residue (fraction III) consisted of stromal remnants and morphologically intact nuclei.

Step 3: extraction of enzyme with 0.15 M KCl. The residue (fraction III) was gelled by bringing the volume to 100 ml with chilled, de-ionized distilled water and by refrigerating the mixture overnight. Sufficient solid KCl was then added to make a 0.15 M KCl solution. This mixture was homogenized for 10 minutes in a Waring blender. The homogenate was centrifuged at 500 G for 30 minutes and the turbid, straw-colored, supernatant solution (fraction IV), was removed for assay of enzyme activity. The inactive precipitate was discarded.

An alternative method for preparing an active enzyme extract from the stromal-nuclei residue (fraction III) was as follows (Steps 3A and 4A).

Step 3A: preparation of acetone powder. Two hundred ml of acetone at -60° C was added slowly, with continuous mixing, to 25 ml of washed, stromal-nuclei residue (fraction III). The mixture was centrifuged at 1,000 G for 10 minutes at -20° C and the supernatant solution was discarded. After the precipitate was washed 3 times with 200 ml of acetone at -60° C, it was suspended in cold acetone and filtered through Whatman no. 1 filter paper in a Büchner funnel. The precipitate was washed with 150 ml of ethyl ether at -60° C and air-dried. The resulting powder (fraction V) was desiccated over concentrated sulfuric acid and stored at 4° C. The yield was 1.25 g.

Step 4A: extraction of the enzyme with Tween-20 and 0.15 M KCl. Acetone powder (fraction V), 2.25 g, was

suspended at 4° C in 100 ml of 0.005 M Tris buffer, pH 7.8, and 0.4 ml of Tween-20 was added. The suspension was mixed intermittently for 30 minutes and allowed to stand overnight at 4° C. Solid KCl, 1.12 g, was added and the mixture was homogenized for 10 minutes in a pre-cooled Waring blender. The homogenate was centrifuged at 500 G at 4° C for 30 minutes, and the slightly turbid, straw-colored supernatant solution (fraction VI) which contained the enzyme activity, was separated from the inactive precipitate. A preparation containing an equal concentration of Tween-20 but without any enzyme was inactive.

RESULTS

Studies on the various enzyme fractions

Recombination of heme synthetase fractions with fraction II. When either enzyme fraction IV or VI was assayed alone, and when fraction II was assayed alone, the amount of heme synthesized was small (Table I). However, the addition of fraction II to either enzyme fraction IV or VI resulted in considerable augmentation of heme synthesis. In eight experiments with different enzyme solutions, a two- to tenfold augmentation of enzyme activity was observed after the recombination of fraction II with either enzyme fraction IV or VI. Therefore, in subsequent experiments, the assay procedure was modified so that 1 ml of fraction II was added to each reaction mixture.

Specific activities of heme synthetase fractions. The results obtained in two typical fractionations are shown in Table II. When the specific activities of enzyme fractions IV and VI were compared in separate experiments with the specific activities of the whole blood hemolysates from which the enzyme fractions IV and VI had been

TABLE I
Effect of recombination of heme synthetase fractions with fraction II*

	Enzyme	Fraction II	Uptake Fe ⁵⁹
	ml	ml	%
Fraction IV	5	0	2.3
	0	1	0.0
	5	1	6.7
Fraction VI	5	0	3.4
	0	1	0.3
	5	1	10.3

* Assay mixtures contained enzyme fractions and/or fraction II as indicated, 0.5 μ mole protoporphyrin, 0.13 μ c ferrous (Fe⁵⁹) citrate, 10 μ moles cysteine, and 1,000 μ moles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

TABLE II
*The specific activities of heme synthetase fractions **

Fraction	Volume	Uptake Fe ⁵⁹	Amt. of N assayed	Specific activity†	Total N	Total units	Yield
	ml	%	mg	U	mg		%
I Hemolysate	100	2.9	113.5	2.6	2,270	5,902	100
II Supernatant solution	125	0.0	10.1	0.0	1,262	0	0
IV 0.15 M KCl enzyme extract	95	17.0	1.0	1,700	19	32,300	540
I Hemolysate	180	7.6	79.42	10	2,859	27,446	100
II Supernatant solution	225	0.0	7.1	0	1,598	0	0
VI Tween-0.15 M KCl enzyme extract	48	20.8	1.64	1,268	16	20,288	73

* Assay mixtures contained 5 ml of hemolysate, 1 ml of fraction II or 5 ml of enzyme preparation (fractions IV or VI) with 1 ml of fraction II, 0.5 μ mole protoporphyrin, 0.13 μ c ferrous (Fe⁵⁹) citrate, 10 μ moles cysteine, 1,000 μ moles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

† Specific activity (units) = 100 \times per cent uptake Fe⁵⁹ per 30 minutes per mg N.

prepared, a 654-fold and a 127-fold purification, respectively, were noted. The total units of enzyme in fractions IV and VI were high. A 5.4-fold increase in total enzyme units of fraction IV as compared to the units in the hemolysate (fraction I) was observed. This is probably because the measurement of enzyme activity in the hemolysate reflects, only in part, the amount of enzyme present, since the enzyme is located in the particulate fraction of the hemolysate (5). Although a further two-fold purification of fraction VI was

achieved by a 35 to 45 per cent ammonium sulfate fractionation, the percentage yield was so low that this step offered little additional advantage in further purification.

Properties of heme synthetase. The effect of temperature, dialysis, and storage at either 5° or – 5° C on the enzymic activity of fraction IV was studied. The enzyme was heat-labile and non-dialyzable (Table III). When fraction IV was stored at either 5° or – 5° C for 2 weeks, there was complete loss of enzyme activity. However, enzyme fraction VI, prepared from an acetone powder (fraction V) which had been stored at 5° C for 4.5 months, was active. Unfortunately, it cannot be stated whether or not there was any decrease in activity during storage, since the acetone powder was not assayed with the same fraction II before as well as after storage.

Studies on the factor in fraction II

Properties. The effect of temperature, dialysis, and storage at either 5° or – 5° C on the factor in fraction II was studied. This factor was heat-labile and nondialyzable (Table III). After storage for 3 months at either 5° or – 5° C, the factor in fraction II was still effective in augmenting heme synthesis.

Effect of globin. The preliminary characterization described above suggested that the factor in fraction II was a protein. Therefore, a series of

 TABLE III
*The effect of temperature and dialysis **

Conditions	Per cent of original activity	
	Fraction II	Fraction IV
37° C	100	100
65° C	80	0
70° C	41	0
80° C	13	0
95° C	0	0
Undialyzed	100	100
Dialyzed	90	97

* Fractions II and IV were heated for 30 minutes or dialyzed for 24 hours against 1 L of 0.005 M Tris buffer, pH 7.8, with five changes of dialysate. Enzyme preparations contained either 1 ml of heated or dialyzed fraction II and 5 ml of fraction IV, or 5 ml of heated or dialyzed fraction IV and 1 ml of fraction II. In addition, assay mixtures contained 0.5 μ mole protoporphyrin, 0.13 μ c ferrous (Fe⁵⁹) citrate, 10 μ moles cysteine, and 1,000 μ moles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

TABLE IV
*The effect of globin **

Enzyme (chicken) fraction IV	Preparations added	Amt.	Uptake Fe ⁵⁹
ml			%
0	Fraction II (chicken)	1 ml	0.0
5	None		0.7
5	Fraction II (chicken)	1 ml	4.5
5	Fraction II (human)	1 ml	4.3
5	Serum (chicken)	1 ml	0.2
5	Serum (human)	1 ml	0.0
5	Albumin (human)	0.16 μ mole	0.3
5	Gamma globulin (human)	0.16 μ mole	0.7
5	Transferrin (human)	0.16 μ mole	0.2
0	Globin (chicken)	0.16 μ mole	0.2
5	Globin (chicken)	0.16 μ mole	2.1
0	Globin (human Hb A)	0.16 μ mole	0.2
5	Globin (human Hb A)	0.16 μ mole	1.9
5	Globin (human Hb SC)	0.16 μ mole	2.3
5	Globin (human Hb G)	0.16 μ mole	2.6

* Assay mixtures contained enzyme fraction IV and/or protein preparations as indicated, 0.5 μ mole protoporphyrin, 0.13 μ c ferrous (Fe⁵⁹) citrate, 10 μ moles cysteine, and 1,000 μ moles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

assays with enzyme fraction IV was performed in which chicken and human sera, purified human serum fractions, and chicken and human globins were substituted for fraction II (Table IV). Augmentation of enzyme activity occurred with globin prepared from chicken hemoglobin and human hemoglobins A, SC, and G; however, no effect could be demonstrated with whole serum or with albumin, γ -globulin or transferrin.

The effect of globin concentration on heme synthesis was studied in a separate experiment. Various amounts of lyophilized chicken globin were dissolved in 1 ml of de-ionized distilled water,

TABLE V
*The effect of globin concentration on heme synthesis **

Enzyme fraction IV (chicken)	Preparations added		Per cent uptake Fe ⁵⁹
ml	Fraction II (chicken)	Globin (chicken)	
	ml	μ moles	
0	1		0.0
5	0		2.6
5	1		8.2
5		1 $\times 10^{-3}$	2.8
5		1 $\times 10^{-2}$	3.3
5		5.6 $\times 10^{-2}$	5.5
5		1 $\times 10^{-1}$	6.5
5		1.6 $\times 10^{-1}$	8.5
5		3.2 $\times 10^{-1}$	7.7
5		5.6 $\times 10^{-1}$	5.6
5		1	3.1

* Assay mixtures contained 5 ml of enzyme fraction IV, 1 ml of a globin chloride solution, 0.5 μ mole protoporphyrin, 0.13 μ c ferrous (Fe⁵⁹) citrate, 10 μ moles cysteine, and 1,000 μ moles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. Incubations were for 30 minutes at 37° C.

added to 5-ml aliquots of enzyme fraction IV, and the mixtures were assayed (Table V). Maximal synthesis occurred when 1 ml of a solution containing 0.16 μ mole globin was substituted for 1 ml of fraction II. At higher globin concentrations there was less augmentation of enzyme activity. Control mixtures of globin, assayed with 5 ml of 0.15 M KCl instead of enzyme, gave less than a 0.4 per cent uptake of Fe⁵⁹. All of the reaction mixtures remained at pH 7.8 during incubation.

One-ml aliquots, containing 0.16 μ mole of an active globin preparation, which had been lyophilized and stored at 4° C, were assayed every 3 weeks with 5 ml of fresh enzyme fraction IV. After storage for 3 weeks, the globin was only 15 per cent as effective in stimulating heme synthesis as the fresh globin. After storage for 6 weeks, the globin failed to stimulate heme synthesis.

Characterization of the reaction product. The observation that globin could be substituted for the factor in fraction II suggested that its role in this reaction might be to bind with the newly synthesized heme to form hemoglobin. To determine whether hemoglobin or heme was the end-product of the reaction, the radioactivity of hemoglobin, isolated by starch block electrophoresis was studied. Reaction mixtures (Table VI), which had been allowed to proceed for 240 minutes to obtain maximal incorporation of label, were centrifuged at 10,000 G, and dialyzed against 0.05 M barbital buffer, pH 8.6, or 0.05 M phosphate buffer, pH 6.8. Electrophoresis of 0.3-ml aliquots was then carried out at pH 8.6 and 6.8, respectively.

TABLE VI
*Recovery of radioactive hemin in the hemoglobin fraction **

Electrophoresis	Total hemin radioactivity		Per cent of hemin radioactivity in Hb
	Before	After	
	$cpm \times 10^{-3}$		
pH 8.6	51.5	51.7	100
pH 6.8	54.4	40.2	74

* Assay mixtures contained 5 ml of enzyme fraction IV, 1 ml of fraction II, 0.5 μ mole protoporphyrin, 0.13 μ c ferrous (Fe⁵⁹) citrate, 10 μ moles cysteine, and 1,000 μ moles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. The incubations were for 240 minutes at 37° C, and the reaction was stopped with 1 ml of 0.1 M potassium cyanide. Starch block electrophoresis was performed in 0.05 M barbital buffer, pH 8.6, and in 0.05 M phosphate buffer, pH 6.8, at 400 v for 4 and 16 hours, respectively.

The hemoglobin, when electrophoresed at pH 8.6 for 4 hours, remained at the origin with a leading edge toward the anode. When electrophoresed at pH 6.8 for 16 hours, the hemoglobin separated into three bands which moved toward the cathode. When a solution of heme was electrophoresed at either pH, the heme migrated rapidly to the anode. After electrophoresis, the starch blocks were cut into five sections so that one section contained all the visible hemoglobin. The samples were eluted with two buffer washes of 7 ml total volume. Carrier hemoglobin was added to each eluate; the heme was crystallized in order to remove the non-heme radioiron, the radioactivity of the hemin was counted, and the total hemin radioactivity was determined.

One hundred per cent of the radioactive heme was recovered with the hemoglobin (Table VI) after electrophoresis at pH 8.6. Seventy-four per cent of the radioactive heme was recovered with the hemoglobin after electrophoresis at pH 6.8. None of the other sections of the starch block contained significant radioactivity.

In a similar experiment, the radioactivity of hemoglobin isolated by chromatography on an IRC-50 column was investigated. To eliminate the possibility that the heme was bound to a protein other than hemoglobin but with the same isoelectric point, a hemoglobin SC hemolysate (18) was used instead of chicken fraction II in the reaction mixture. The reaction mixture was chromatographed and the minor hemoglobin components (14) and the major hemoglobin S and hemoglobin C components were separated. As the column was developed, the hemoglobin concentration, as measured by absorbance at 415 m μ , and the radioactivity were determined in each milliliter of eluate. The amount of radioactive heme present in pooled samples of from 1 to 4 ml, depending on the radioactivity, was then determined.

When the radioactivity of the heme and the concentration of hemoglobin were plotted on the same axis, the peaks of radioactivity and hemoglobin coincided (Figure 1). The specific activities, expressed as the hemin radioactivity (cpm) per unit of absorbancy for each hemoglobin fraction, were as follows: minor hemoglobin components, 158 cpm; major hemoglobin S, 148 cpm; and major hemoglobin C, 173 cpm. Similar labeling of the minor and major hemoglobin components oc-

curred in an experiment in which a hemoglobin A hemolysate was used.

DISCUSSION

In the experiments presented (Table II), when the specific activities of enzyme fractions IV and VI were compared with those of the hemolysates from which they had been prepared, a 654-fold and a 127-fold purification, respectively, were noted. Similar preparations of fraction VI from different hemolysates have varied from 50- to 900-fold in the purification of enzyme from the hemolysate. Fraction IV, like previously described enzyme preparations (5-7), was unstable. However, enzyme stored as an acetone powder (fraction V) was stable. This suggests that the preparation of an acetone powder (fraction V) would be a preferable procedure in further purification studies.

The purification of the heme synthetase system was facilitated by the observation that a heat-labile, nondialyzable, stable factor present in the supernatant solution (fraction II) of the original hemolysate was required, in addition to the enzyme, for maximal synthesis of heme. Although human nonreticulated cells are not capable of synthesizing heme (3), fraction II, prepared from human cells, was as effective as chicken fraction II in augmenting the activity of purified heme synthetase. Similar observations have been made by Minakami, Yoneyama and Yoshikawa (19). Rabinovitz and Olson (20) have reported that the incorporation of iron into hemoglobin by a rabbit reticulocyte microsomal system requires a soluble fraction.

The effect of globin accounts in part, if not entirely, for the effect of fraction II on the synthesis of heme. Globin was prepared five times by the method of Rossi-Fanelli, Antonini and Caputo (12), and four of the preparations stimulated heme synthesis. With two of these preparations the incorporation of radioiron into heme was as great in the presence of optimal amounts of globin as in the presence of fraction II. When globin was prepared five times by the method of Anson and Mirsky (21), four of the preparations also stimulated heme synthesis. With none of these preparations was the incorporation of radioiron into heme as great as in the presence of fraction II. This variability in the effectiveness of different preparations of globin to stimulate heme synthesis and

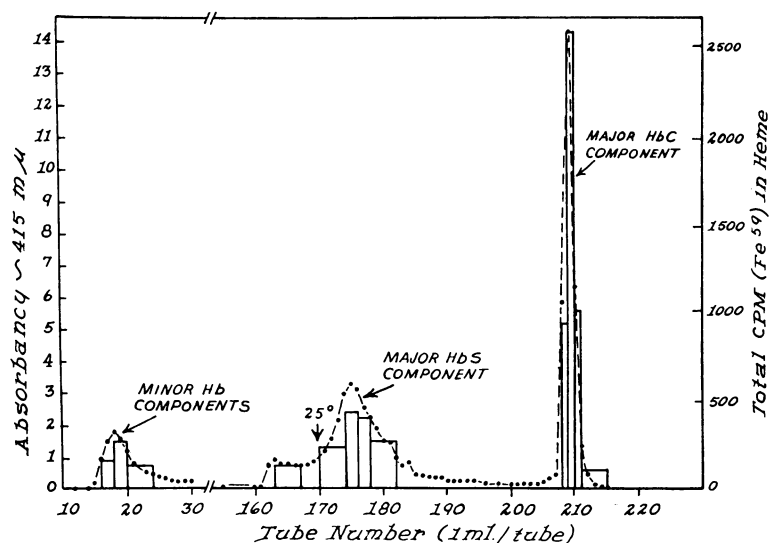


FIG. 1. THE CHROMATOGRAPHIC SEPARATION OF RADIOACTIVE HEMOGLOBINS S AND C. The radioactivity of the heme (columns), expressed as total cpm in the heme, and the concentration of hemoglobin (broken line), expressed as absorbancy at 415 $m\mu$, are compared. The assay mixture contained 5 ml of enzyme fraction VI, 1 ml of a hemoglobin SC hemolysate, 0.5 μ mole protoporphyrin, 0.13 μ c ferrous (Fe^{59}) citrate, 10 μ moles cysteine and 1,000 μ moles Tris buffer, pH 7.8, in a final volume of 10 ml with 0.15 M KCl. The incubation was for 240 minutes at 37° C, and the reaction was stopped with 1 ml of 0.1 M potassium cyanide. There was 55.1 per cent uptake of Fe^{59} . In a similar assay mixture, which did not contain enzyme, there was 0.6 per cent uptake of Fe^{59} . Chromatography was on a 1 \times 35 cm IRC-50 column which was developed at 5° C with phosphate buffer, pH 7 (Developer no. 2). The buffer was 0.0625 M in sodium ion. At tube no. 170, the column was warmed to 25° C. The distortion seen in the hemoglobin S peak was produced by the emergence of some of this hemoglobin before the column warmed to 25° C.

the failure to observe an effect with stored globin preparations are consistent with the known instability of purified globin preparations (22) and may explain why other investigators have not observed an effect of globin on heme synthesis (23).

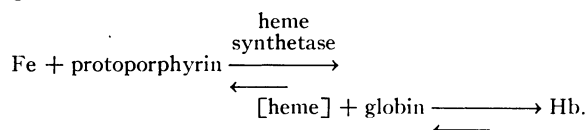
The recovery of most, if not all, of the radioactivity in the heme from the hemoglobin isolated by electrophoresis or chromatography can be taken as evidence that the end-product of the reaction was hemoglobin and not free heme. In this regard it is interesting that the observed (Table V) optimal ratio of globin concentration (0.16 μ mole per 10 ml reaction mixture) to protoporphyrin concentration (0.5 μ mole per 10 ml reaction mixture) was 1.2:4. This ratio is in good agreement with the expected 1:4 ratio, i.e., four heme groups per molecule of hemoglobin. Furthermore, when different hemoglobin components from a reaction

mixture were separated by chromatography, it was found that the labeling of the hemoglobin was of similar specific activity whether globin from hemoglobins A, S, C or from minor components was available. Similar observations were made by Minakami and associates (19) when the enzymic incorporation of radioiron into horse and duck hemoglobins was studied. The abnormal hemoglobins S, C and G have been shown to differ from hemoglobin A in their primary structure (24, 25); however, globins prepared from these hemoglobins were as effective as chicken globin in stimulating heme synthesis.

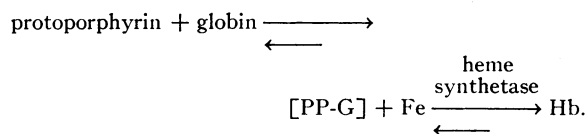
Although the nonenzymic formation of heme has been observed at neutral pH in the presence of protoporphyrin, iron, globin and a detergent (26), we have not been able to detect such a reaction under the conditions of our experiments. In the

present studies, globin, in addition to cysteine, or reduced glutathione, was shown to be required for maximal synthesis of heme from iron and protoporphyrin by the heme synthetase system. The observed effect of globin and the lack of effect of other proteins on the synthesis of heme suggests that the globin effect is specific. However, since the enzymic synthesis of heme is probably the same in all tissues (27-29), one might expect that other heme apoproteins will show an effect similar to that of globin.

The present studies do not elucidate the exact mechanism of hemoglobin formation from iron, protoporphyrin and globin. The heme, which is synthesized enzymically, may bind with globin (30), drawing the reaction to the right as hemoglobin is formed:



Presumptive evidence in favor of this mechanism is the recent demonstration by Rossi-Fanelli and Antonini (31) that the heme groups of heme proteins exist in equilibrium with free heme and can exchange with other apoproteins. On the other hand, the globin may act in the formation of a protoporphyrin-globin complex (PP-G) prior to the enzymic incorporation of iron (32):



Elucidation of the mechanism of these reactions will have to await the isolation of the pure enzyme and subsequent kinetic studies.

SUMMARY

1. The formation of hemoglobin from iron, protoporphyrin and globin was studied in a chicken hemolysate system. Heme synthesis was measured as the per cent incorporation of added radioiron into heme.

2. The heme synthetase enzyme system was purified several hundredfold. An acetone powder of a purified enzyme preparation was stable for 4.5 months at 5° C.

3. A factor present in the supernatant solution (fraction II) from a chicken or human hemolysate was required for maximal incorporation of radioiron into heme.

4. The factor present in fraction II could be replaced by optimal concentrations of chicken globin. A similar effect was observed with human globin.

5. The end-product of the reaction, as characterized by electrophoresis and chromatography, was hemoglobin.

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