AN INTRACELLULAR PROTEIN INTERMEDIATE FOR HEMOGLOBIN FORMATION *

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Iron and amino acids enter young red cells and are incorporated into hemoglobin, but the pathway of iron after entering the erythroblast is not understood nor is it clear in what sequence iron, protoporphyrin, and globin join to form a complete hemoglobin molecule. Several lines of evidence have suggested the presence of an intracellular, nonheme iron protein that may be an intermediate in the formation of hemoglobin (1-10). This report describes a protein fraction from human and dog marrow cells which appears from isotopic studies to be such a precursor of hemoglobin. It does not contain heme, and it can be readily differentiated from siderophilin and ferritin.

MATERIALS AND METHODS

General methods. Serum iron and iron-binding capacity were determined by the method of Peters, Giovanniello, Apt and Ross (11). Quantitation of hemoglobin samples was done on a Beckman model DU spectrophotometer. Molecular extinction coefficients used were 1.2×10^5 at 415 $m\mu$ for oxy- or carbonmonoxy-hemoglobin and 1.08×10^5 at 415 $m\mu$ for cyanmethemoglobin. Other proteins were determined by extinction at 280 $m\mu$ or by a turbidometric method with cationic detergent and alkali (12). Heme was isolated by the method of Fischer (13) or by the method of Kassenaar, Morell and London (14).

Isotopes and counting. Uniformly labeled L-leucine- C^{14} (4.15 mc per mmole) was purchased from Merck and Co., Montreal; glycine-2- C^{14} (2.44 mc per mmole) from New England Nuclear Corp.; and Fe^{59} -citrate (approx. 500 mc per mmole) from Abbott Laboratories.

Radioactive iron was counted in a well scintillation counter (Baird-Atomic model 810 detector with 1.75×2 inch crystal, modified to match Packard Autogamma

spectrometer, series 410A). Efficiency for Fe^{59} was about 0.12. C^{14} counting was performed on a Robinson windowless flow-gas counter (15) or on a Picker thin-window flow counter (2975A) with automatic sample changer. Efficiency was about 20 per cent. C^{14} samples were plated at infinite thinness on platinum discs as liquids and dried under a heat lamp.

Marrow procurement. Marrow was obtained from the ribs of hematologically normal human beings undergoing thoracotomy, or from the long bones of dogs 6 to 12 months of age, and suspended in Hanks' solution containing autologous serum. The marrow was sieved through a stainless steel tea strainer and then passed through a stainless steel screen with openings 200 μ square (16). This procedure resulted in a well dispersed cell suspension suitable for accurate pipetting and cell counts. The marrow was processed and used within 1 hour after it was removed from the body.

Incubation of intact marrow cells. Incubations were carried out at 37.5° C in air with gentle shaking in Erlenmeyer flasks with an air:liquid ratio greater than 8:1. Acid-cleaned, siliconed glassware was used throughout. The incubation medium was serum in Hanks' solution (see legends to figures), buffered to pH 7.6, and contained 10,000 U penicillin, 60 μ g streptomycin, and 5 mg glucose per ml. At the start of incubation, radioactive substrates were added. In the case of iron the quantities added were always less than the binding capacity of the serum present. Reactions were stopped by the addition of 1 per cent KCN and 2 per cent $K_3Fe(CN)_6$ with rapid cooling to 0° C.

Preparation of hemoglobin solutions. After incubation the cells were washed 3 times with 10 vol isotonic saline and centrifuged for 10 minutes at 240 G. The cells were lysed by exposure to 4 vol distilled water for 15 minutes at 4° C with vigorous shaking. The lysate was centrifuged for 15 minutes at 1,500 G at 4° C, and the supernate was then centrifuged for 30 minutes at 100,000 G at 4° C. The resulting clear red "hemoglobin solution" was kept at 4° C until chromatographed, usually within 24 hours.

Hemoglobin solutions stored for long intervals at 4° C, at room temperature, or frozen at -20° C gave altered chromatograms (17). Dialysis of hemoglobin solutions was associated with the formation of precipitates, and hence dialysis was not usually performed. In some instances brief dialysis was necessary to obtain adsorption to the resin.

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Incubation of lysed marrow cells. Marrow cells were washed twice in Hanks' solution by centrifuging for 10 minutes at 375 G. Four vol of distilled water was added to the cell sediment, and the mixture was shaken at 4° C for 15 minutes. Lysis was stopped by the addition of an appropriate volume of a fivefold concentrate of an intracellular buffer of pH 7.9. The composition of the diluted buffer in milliequivalents per liter was K^+ , 150; Mg^{++} , 10; Cl^- , 130; HCO_3^- , 30. The lysate was centrifuged for 15 minutes at 1,500 G, and aliquots of the clear supernate were pipetted into incubation flasks. At the end of incubation, reactions were stopped with KCN and $K_3Fe(CN)_6$ as before, particulate matter was removed by centrifuging at 100,000 G for 30 minutes, and the samples were dialyzed overnight against distilled water prior to chromatography.

Chromatography on IRC-50. Amberlite IRC-50, XE-64, was obtained from Rohm and Haas Co. IRC-50 was prepared according to Hirs, Moore and Stein (18) and was titrated from the acid side to near the desired pH with NaOH. The resin was then washed with a 100 vol excess of distilled water and equilibrated with the starting buffer until the pH was regulated to within 0.05 pH U of pH 7.00. Hemoglobin binding varies greatly with very small pH changes between pH 6.8 and 7.2, and accurate equilibration of a batch of resin is critical. Columns (1×10 cm) were generally packed by gravity 1 to 2 hours before use.

We evolved a linear gradient elution system using two identical 500-ml cylindrical bottles connected at the bottom by polyethylene tubing. The starting buffer was 0.0067 M sodium phosphate with 0.0033 M KCN at pH 7.0. The limiting buffer was 0.5 M NaCl dissolved in the starting buffer. Flow rates of 1 to 2 ml per cm^2 per hour were used. The rate of increase in ionic strength was regulated by the volumes placed in the reservoirs, as well as by the flow rates.

Initial investigation of rates of incorporation of labeled amino acids and iron into the various components separated by IRC-50 columns showed no difference except for the fraction that elutes with the solvent front. This fraction (Fr I), termed V_0 by Huisman and Prins (19), showed a markedly different kinetic pattern and specific activity. Accordingly, in many experiments Fr I was separated with the starting buffer, and all other components were eluted from the column by changing directly to the limiting buffer.

Recoveries from IRC-50 columns were better than 95 per cent, based on measurements of absorbance of 280 and 415 $m\mu$. Fractions were collected on a volume-activated fraction collector or, in the case of the two-stage columns, by hand.

Chromatography on DEAE-cellulose. This system was used for the further purification of Fr I. DEAE-cellulose (Selectacel, Brown Co., Berlin, N. H.) was alternately washed with 0.1 N HCl and 0.1 N NaOH three times, ending with an alkaline cycle, and then repeatedly washed with distilled water to pH 7.0. A linear gradient of increasing ionic strength and decreasing pH was used, starting with 300 ml distilled water. The limiting buffer

was 300 ml 0.01 M acetate at pH 4.0 with 2 M NaCl. Columns of 40-cm length and 1-cm diameter were used and were packed by gravity.

Immunochemical studies. Antibodies were made in female rabbits against dog liver ferritin and dog Fr I. The ferritin was made according to Granick (20) and was recrystallized three times. Fr I was purified by DEAE-cellulose chromatography as described above. Rabbits were injected with 9 mg of Fr I mixed with Freund's complete adjuvant (Difco-Bacto); 12 to 16 injection sites in the neck and sacrospinalis muscle group were used, each depot being less than 0.5 ml. Thirteen mg of ferritin was used in similar fashion. At 4 weeks the rabbits were exsanguinated by heart puncture and the serum harvested. Antibody activity was tested by a precipitin ring test against dilutions of antigen. The antiferritin antiserum produced precipitation with 2 μg of ferritin per ml; the anti-Fr I with 23 μg of Fr I per ml. Antisiderophilin was a sample of goat antihuman transferrin, kindly provided by Hyland Laboratories.

Concentration of Fr I. This was done to some extent by dialysis against polyvinylpyrrolidone. Considerable loss due to the formation of an insoluble precipitate limited efforts at concentration.

Electrophoresis. Electrophoresis was performed on cellulose acetate (Oxoid) in a Shandon Universal electrophoretic apparatus (Consolidated Laboratories, Inc., Chicago Heights, Ill.); 5×12 to 18 cm strips were used in a 0.005 M veronal buffer, at pH 8.6, with 13 v per cm. After 2 hours the strips were fixed and stained with Lissamine green SF for protein, as described by Brackenridge (21).

RESULTS

Chromatography. Figure 1 is a typical chromatogram of a marrow cell lysate. Fr I, although

FIG. 1. IRC-50 CHROMATOGRAM OF NORMAL HUMAN MARROW LYSATE DEVELOPED BY A LINEAR GRADIENT AT pH 7.0. Details are given in Methods.

heterogeneous, consisted mainly of nonheme proteins, while the subsequent fractions were made up of hemoglobins. After incubation of marrow *in vitro* with Fe^{59} - or with C^{14} -labeled amino acids, the relative activities of these hemoglobins were similar at all incubation times, while the activity of Fr I was markedly higher. In the following discussion the hemoglobins (fractions 20 to 80) are considered collectively and referred to as "Main Hb."

Kinetics. Figure 2 illustrates the incorporation rate of Fe^{59} into Fr I and Main Hb. A significant amount of Fe^{59} appeared in the Main Hb only

FIG. 2. INCORPORATION OF Fe^{59} BY FR I AND MAIN HB WITH INCREASING TIME OF INCUBATION. Human rib marrow (96×10^6 nucleated cells per ml) was incubated with Fe^{59} ($3.6 \mu\text{c}$ per ml) in 50 per cent serum in Hanks' solution.

after a delay of 7 to 10 minutes. Thereafter hemoglobin synthesis proceeded at a linear rate for 4 to 6 hours. There was no delay in the appearance of radioactivity in Fr I, and its activity rose steeply to peak values in 10 to 30 minutes.

Figure 3 shows the incorporation rate of L-leucine- C^{14} into Fr I and Main Hb. The incorporation of leucine- C^{14} into Fr I and Main Hb showed the same time curves as that of Fe^{59} . There was no significant difference between human and dog marrow with respect to the kinetics of Fe^{59} - or C^{14} -labeled amino acid incorporation. After incubation *in vitro* there was a wide varia-

FIG. 3. INCORPORATION OF C^{14} -LEUCINE INTO FR I AND MAIN HB. Human rib marrow (75×10^6 nucleated cells per ml) was incubated with C^{14} -leucine ($0.4 \mu\text{c}$ per ml) in 50 per cent serum in Hanks' solution.

tion from one marrow to another in the degree of labeling of hemoglobin. This variation was still observed after correction for the number of red cell precursors present and any differences in the substrate. Different sera have widely differing potency in promoting heme synthesis (16). For this reason a direct comparison between different experiments is difficult.

Figure 4 illustrates the ratio of Fe^{59} incorporation into Main Hb and Fr I at progressive times in all experiments performed. Despite different absolute levels of synthesis, the ratios of Main

FIG. 4. THE RATIO OF Fe^{59} INCORPORATION INTO MAIN HB AND FR I AT PROGRESSIVE TIMES OF INCUBATION IN ALL EXPERIMENTS PERFORMED.

FIG. 5. INCORPORATION OF Fe^{59} INTO FR I AND MAIN HB. Human rib marrow (133×10^6 nucleated cells per ml) was incubated with Fe^{59} ($1.06 \mu\text{C}$ per ml) in 33 per cent serum in Hanks' solution. After 10 minutes the cells were washed, resuspended in the same medium with unlabeled Fe, and reincubated for 4 hours.

Hb to Fr I were similar. In the first 10 minutes the total Fe^{59} in Fr I was two to ten times that in the Main Hb. The activities became equal after about 30 minutes, and thereafter there was a progressive increase in that of Main Hb.

Effect of lead. Lead acetate (10^{-4} M) added to either dog or human marrow incubations blocked the incorporation of iron into Main Hb. There was no inhibition of Fe^{59} incorporation into Fr I. Lead added after peak values for Fr I had been achieved produced no significant change in the activity of Fr I, although Main Hb synthesis was diminished.

Reincubation in unlabeled substrate. It seemed likely on the basis of the kinetic pattern that Fr I contained an iron compound, possibly an iron protein, that was an intermediate for hemoglobin formation. The rate of total activity entering Fr I per unit time in the first minutes of reaction was enough to explain all activity subsequently appearing in the hemoglobin fraction. To test this possibility further the following experiments were performed (Figures 5 and 6): aliquots of a marrow suspension were incubated for 10 minutes with Fe^{59} bound to autologous serum protein or with leucine- C^{14} . At 10 minutes the incubation

was stopped by lowering the temperature to 4°C . The marrow cells were washed free of external radioactivity at 4°C and reincubated for 4 hours in the same concentration of unlabeled iron or unlabeled leucine as was present initially.

There was a fall in total Fe^{59} activity in Fr I and a rise in activity in the Main Hb which, although less rapid than prior to cell washing, was definite (Figure 5). The stroma, mitochondria, and microsomes also showed a drop in activity after cell washing. That the Fe^{59} in Fr I did not fall further may be due to the large amount of Fe^{59} that became attached to the stroma in these experiments. It appeared possible that Fr I received iron from stroma and yielded it to hemoglobin (8). As long as the stromal iron pool was high, a large drop in Fe^{59} in Fr I would not be expected.

The C^{14} level of Fr I also fell, while the C^{14} level of Main Hb increased (Figure 6). An active turnover of proteins that are not on the pathway of hemoglobin synthesis could account for a greater drop in C^{14} activity of Fr I than appeared in the Main Hb.

Marrow lysate experiments. In order to gain more direct evidence that the active material in Fr I is an intermediate for hemoglobin synthesis,

FIG. 6. INCORPORATION OF C^{14} -LEUCINE INTO FR I AND MAIN HB. Human rib marrow (133×10^6 nucleated cells per ml) was incubated with C^{14} -leucine ($2.5 \mu\text{C}$ per ml) in 33 per cent serum in Hanks' solution. After 10 minutes the cells were washed, resuspended in the same medium with unlabeled leucine, and reincubated for 4 hours.

FIG. 7. INCORPORATION OF LABELED FR I INTO MAIN Hb BY A LYSATE OF DOG MARROW CELLS. In each experiment $0.6 \mu\text{C}$ (270,000 cpm) C¹⁴-leucine was used as the control substrate. In experiment A, the labeled Fr I contained 70,000 cpm; in experiment B, 100,000 cpm. The lysates were incubated for 30 minutes.

incubation of marrow lysates was carried out. The cell-free lysate system had no ability to incorporate leucine-C¹⁴ into Main Hb in either the presence or absence of added Fr I. However, when Fr I which had been previously labeled with leucine-C¹⁴ was incubated with the lysate, there was a prompt and striking appearance of C¹⁴ in the Main Hb (Figure 7).

The lysate system was able to incorporate Fe⁵⁹ from added Fe⁵⁹-citrate into Main Hb (Figure 8). Addition of Fr I enhanced the incorporation appreciably, while addition of serum diminished it. If Fr I previously labeled with Fe⁵⁹ was used as the substrate for the lysate system, Fe⁵⁹ rapidly appeared in the Main Hb. By addition of carrier hemoglobin with subsequent heme crystallization it was shown that the nonheme iron in Fr I had been converted to heme in the Main Hb.

FIG. 8. INCORPORATION OF Fe⁵⁹ INTO MAIN Hb BY A LYSATE OF HUMAN MARROW CELLS. In each experiment $0.71 \mu\text{C}$ (190,000 cpm) of Fe⁵⁹-citrate was added to a 2-ml aliquot of the lysate; 1.4 ml of saline was added to the controls, 1.4 ml of serum to the "serum" aliquot, and 1.4 ml of a solution of "cold" Fr I (1 mg/ml) to the "Fr I" aliquot. The lysates were incubated for 15 minutes.

Characterization of fraction I

Absence of radioactive heme. Fr I showed maximal absorption at $280 \text{ m}\mu$, but did have some absorption at $415 \text{ m}\mu$ (Figure 1). When Fr I labeled with Fe⁵⁹, leucine-C¹⁴, or glycine-2-C¹⁴ was mixed with carrier hemoglobin, the isolated heme contained less than 1 per cent of the activity expected if all Fr I counts were assumed to be in hemoglobin.

Iron binding. The iron-binding affinity of Fr I was tested by dialysis against various buffers. Table I indicates the binding strength of Fr I compared with that of inorganic iron, siderophilin, and

TABLE I

*The relative binding affinity of several Fe⁵⁹-labeled compounds is illustrated by dialysis against various buffers **

	Time in hours	Distilled water	Acetate pH 4.6	Acetate + EDTA pH 4.6	Ascorbic acid pH 2.7	Thioglycollic acid pH 7.2
Fe ⁵⁹ -citrate	2	100	49	22		
in dist. water	24	89	2	<1		
Iron-binding protein (serum)	2			2		
Main Hb fraction (IRC-50)	2		100	100	93	89
	24		100	100	82	55
Fraction I (IRC-50)	2		100	100	90	100
	24		100	100	60	67

* Results are recorded as per cent of original radioactivity remaining in the dialysis bag after the indicated number of hours.

hemoglobin. The iron in Fr I was apparently bound about as firmly as the iron in heme under the conditions employed.

Stability. Clear solutions of Fr I became cloudy in a few hours with subsequent slow formation of a precipitate at 4° C. Room temperature or freezing accelerated precipitate formation, and dialysis usually resulted in appreciable loss. In more recent experiments it was observed that the addition of a reducing agent, such as 0.005 M cysteine, seemed to stabilize solutions of Fr I. Fr I could be heated to 70° C for 15 minutes without precipitation and without alteration of electrophoretic migration. In this case a dark-brownish protein

CELLULOSE ACETATE ELECTROPHORESIS .06 M BARBITAL BUFFER pH 8.6

FIG. 9. ELECTROPHORESIS OF DOG LIVER FERRITIN AND OF Fe^{59} -LABELED CRUDE FR I FROM AN IRC-50 CHROMATOGRAM.

precipitate settled out, and the Fe^{59} radioactivity remained in the supernate.

Distinction from siderophilin. Fr I bound iron much more firmly than did siderophilin (Table I). These two substances exhibited different rates of migration upon electrophoresis on cellulose acetate, at pH 8.6, with veronal buffer. There was no cross reaction of antisiderophilin antiserum with Fr I.

Distinction from ferritin. Fr I and ferritin have in common the property of heat resistance. However, upon electrophoresis the radioactivity in crude Fr I migrated in the opposite direction from ferritin (Figure 9). Crude Fr I placed opposite antiferritin antiserum produced a faint band in agar diffusion, but this band did not coalesce with bands produced by crystalline liver ferritin (Fig-

FIG. 10. AGAR GEL DIFFUSION PLATE. Center well, antidog liver ferritin; 1, crude Fr I; 2, 4, 6, dog liver ferritin; 3, dog hemoglobin; 5, Fr I purified by DEAE-cellulose chromatography.

ure 10). There was no apparent reaction with purified dog Fr I. Anti-Fr I produced no reaction with crystalline liver ferritin.

Purification. A system of DEAE-cellulose column chromatography gave some resolution of the components of dog Fr I. A typical chromatogram is shown in Figure 11. The material in crude Fr I absorbing at 415 $m\mu$ appeared in the first peak. Most of the Fe^{59} coincided with a subsequent sharp maximum of absorption at 280 $m\mu$. This material had a ratio of $D_{415} : D_{280}$ of less than 1:1,000 indicating no detectable heme protein.

FIG. 11. CHROMATOGRAM OF CONCENTRATED DOG FR I ON DEAE-CELLULOSE.

That this purified material is a protein is indicated by the following facts. It has a single absorption maximum at 280 $m\mu$; it is nondialyzable; it is precipitated by 5 per cent trichloroacetic acid; it has the staining properties of a protein on electrophoresis; it reacted with cationic detergent and alkali in the method of Jacox (12) for protein determination.

DISCUSSION

These studies have demonstrated that, when marrow is incubated with Fe^{59} , radioactivity appears in the first few minutes not only in stroma and particulate matter but also in a soluble nonheme protein fraction (Fr I). There is a delay of some 5 to 8 minutes before significant activity appears in hemoglobin. Consideration of total count flux suggests that Fr I must play a major role in iron transport to hemoglobin.

Two pathways for the movement of iron into young red cells have been described: the transfer of ferritin from macrophages to young red cells by pinocytosis (4); and the direct transfer of iron from the plasma iron-binding protein to the young red cells (2, 8). Since marrow is composed of many cell types, our studies cannot exclude a cell-to-cell transfer. However, radioautographic studies indicate that most of the iron taken up by marrow cells is in the red cell precursors (22), and microspectrophotometric studies show nonheme iron in these cells (9). Reticulocyte preparations that do not contain macrophages are capable of making hemoglobin from Fe^{59} bound to plasma iron-binding protein (23). We have shown that the active material in Fr I is neither plasma iron-binding protein nor ferritin. Hence, the weight of evidence indicates that the kinetics described occur entirely within young red cells.

Several investigators have suggested an intracellular intermediate in the transport of iron from stroma to hemoglobin (1, 2, 8, 9). The studies presented here describe an intracellular iron protein that was readily differentiated from siderophilin and ferritin, the only known soluble nonheme proteins important in iron transport. This soluble protein appears to be the vehicle by which iron is moved from fixed stromal pools to hemoglobin. The relationship, if any, of this soluble protein to the "heme synthetase system" and, especially,

"fraction II" described by Schwartz and colleagues (24) remains to be elucidated.

The striking parallel in kinetics between iron and amino acid incorporation raises the possibility that this protein is a precursor of the globin portion as well as of the iron of hemoglobin. This concept is reinforced by the observation of the appearance of C^{14} -leucine in Main Hb when Fr I prelabeled with C^{14} -leucine was incubated in a cell-free marrow lysate. Studies are continuing on the nature of the Fe^{59} and leucine precursor substances.

If the active portion of Fr I is incorporated *in toto* into hemoglobin, it is necessary that the leucine-containing material be closely related to, or identical with, part of the globin molecule. As yet, enough material of sufficient purity is not available to make any comment other than that there are many properties in common between the two. It is interesting to note that a highly labeled protein closely related to globin present in young red cells could well contribute an important increment of radioactivity to both *in vivo* and *in vitro* studies of heme versus globin formation. This problem could be avoided by using an IRC-50 column to prepare "pure" hemoglobin before the isolation of globin.

A scheme of hemoglobin synthesis which includes a common precursor for the iron and the peptide chains may be discussed as outlined in Figure 12. Iron from siderophilin, and perhaps ferritin, enters the hemoglobin-forming cell, where it is bound to the insoluble stromal proteins (8). Globin synthesis, from amino acid or peptides, and protoporphyrin synthesis, via δ -amino levulinic acid and porphobilinogen, occur within the cell. As globin is formed, it, or part of it, combines with the available iron to form the common precursor which, for brevity, might be termed "sideroglobin." Sideroglobin combines with protoporphyrin to form hemoglobin. An alternative pathway, combination of protoporphyrin with iron to form heme, has been more frequently considered (24), but it is of interest that an excess of heme in young red cells, normal or abnormal, has not been described. On the other hand, an excess of free protoporphyrin has been demonstrated in several abnormal states. As indicated in Figure 12, this might be expected to occur from a deficiency of

FIG. 12. A SCHEME FOR HEMOGLOBIN FORMATION SUGGESTED BY OUR EXPERIMENTS. The term "sideroglobin" refers to a combination of iron with a protein, possibly a part of the globin molecule.

iron, a deficiency of globin synthesis, a failure of iron to combine with globin to form sideroglobin, or a failure of the final step, the union of sideroglobin with protoporphyrin to form hemoglobin. An excess of free erythrocyte globin has not been recognized, but its existence would be expected. Other defects, both inherited and acquired, of the final steps of hemoglobin formation should be found and may be better understood in the light of mechanisms depicted in Figure 12.

SUMMARY

Marrow cells from human and dog were incubated for short intervals with radioactive iron or amino acids. The marrow lysate was chromatographed on an IRC-50 column. A minor fraction (Fr I) became labeled before hemoglobin when marrow was incubated with Fe^{59} or C^{14} -leucine. Further studies provided additional evidence that this fraction contained a precursor to hemoglobin. Heme isolated from the fraction was unlabeled. Purification of Fr I by DEAE-cellulose column chromatography showed that Fe^{59} was bound to a nonheme protein distinct from ferritin and siderophilin. These observations suggest the possibility that in the course of hemoglobin formation iron combines with a protein, possibly a part of the globin molecule, which unites with protoporphyrin to form heme and the complete hemoglobin molecule.

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