Mechanism of Hemolysis Induced by Ferriprotoporphyrin IX

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ABSTRACT Incubation of a 0.5% suspension of washed, normal mouse erythrocytes with ferriprotoporphyrin IX (FP) at 37°C and pH 7.4 caused potassium loss, swelling, increased susceptibility to hypotonic lysis, and finally hemolysis. Hemolysis was not inhibited by incubation in the dark, malonyldialdehyde was not produced, and various free radical scavengers had no effect on the hemolysis. Only the sulfhydryl compounds, cysteine, dithiothreitol, and mercaptoethanol protected erythrocytes from FP. Potassium loss reached 90% within 30 min of exposure to 5 μM FP. This amount of FP caused >50% hemolysis within 2.5 h. Sucrose (0.1 M) completely prevented hemolysis but had no effect on potassium loss. Likewise, reducing the temperature from 37 to 25°C greatly retarded hemolysis but had no effect on potassium loss. These observations indicate that FP impairs the erythrocyte's ability to maintain cation gradients and induces hemolysis by a colloid-osmotic mechanism.

INTRODUCTION

Heme loss occurs spontaneously from certain abnormal hemoglobins (1–3) and can be induced by oxidation of the sulfhydryl groups of normal hemoglobin (3, 4). After heme loss, Heinz bodies precipitate (1–3), and the affected erythrocytes exhibit abnormal cation flux (3) and reduced deformability (5). The possibility that Heinz bodies may cause hemolysis has been studied extensively (6, 7). By comparison, little consideration has been given to the possibility that the liberated heme moiety may cause hemolysis. When heme is liberated from hemoglobin in the presence of oxygen, it is released as ferriprotoporphyrin IX (FP)[1] (8). We have found previously that FP is a potent hemolytic agent (9). This work was undertaken to define the mechanism of hemolysis by FP.

METHODS

Male, Swiss-Webster mice, weighing 20–25 g, were used as donors of normal erythrocytes. The erythrocytes were washed three times as described (10) using a standard medium which also has been described (11); it has an osmolality of 275 mosmol and is buffered to pH 7.4 with 50 mM phosphate. FP (hemin from equine blood) was purchased from Sigma Chemical Co. (St. Louis, Mo.) and was recrystallized once by the method of Labbe and Nishida (12). On the day of each experiment, a fresh stock solution of 0.5 mM FP was prepared in 5 mM NaOH and placed in an ice bath. Immediately before use, an aliquot of the stock solution was diluted to the appropriate concentration using ice-cold standard medium; after dilution the pH of FP-containing solutions was 7.4.

The effect of FP on erythrocytes was evaluated by measuring hemolysis, erythrocyte swelling, osmotic fragility (13), potassium loss, lipid peroxidation, and ATP (14) and glutathione (15) concentrations. In the studies of hemolysis, erythrocytes were incubated in plastic vessels in an Eberbach shaking incubator operating at 2.3 Hz. At the end of incubation, intact erythrocytes were sedimented by centrifugation, and the amount of hemoglobin in the supernatant solution was determined by measuring the optical density at 540 nm. Then the erythrocyte pellet was lysed with distilled water, and its hemoglobin content was measured. Percent hemolysis was calculated from these measurements.

Potassium loss was evaluated using standard medium that had been modified by replacing KCl with an equimolar amount of NaCl. To measure potassium loss after various periods of time, the incubation mixtures were diluted with an equal volume of potassium-free medium, the erythrocytes were collected by centrifugation, and the erythrocyte pellet was extracted with 0.5 M perchloric acid for measurement of potassium with a flame photometer. The results of these measurements and of measurements of the amounts of potassium in the erythrocytes at the beginning of incubation were used to calculate the percent loss of cellular potassium.

The extent of lipid peroxidation in erythrocytes treated with FP was evaluated by measuring malonyldialdehyde, using the thiobarbituric acid test. The thiobarbituric acid reagent was prepared as described by Kohn and Liversedge (16). For the color reaction, one part of a cell suspension was mixed with 1 part of 10% (wt/vol) trichloroacetic acid and

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[1] *Abbreviations used in this paper*: FP, ferriprotoporphyrin IX.

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2 parts of 0.67% thiobarbituric acid reagent. This mixture was heated for 15 min at 55–60°C, then immediately cooled in an ice bath and centrifuged. The malonyldialdehyde-thiobarbituric acid complex in the supernatant solution was determined by measuring OD at 534 nm.

RESULTS

The concentration, time, and temperature dependencies of the hemolytic effect of FP are shown in Figs. 1 and 2. Only mouse erythrocytes were used in the present experiments, but a preliminary evaluation of human erythrocytes indicated that they are affected similarly by FP. In agreement with previous work with mouse erythrocytes, 5 μM FP was more than sufficient to cause 50% hemolysis during a 2.5-h incubation period at 37°C and pH 7.4 (9). Also in agreement with the earlier work (9), the time-course of hemolysis revealed the existence of at least two phases. With 5 μM FP there was a lag phase of ~1 h. Increasing the concentration of FP abbreviated this phase (Fig. 1). After the lag phase, rapid hemolysis occurred. Changing the exposure to light did not affect the course of hemolysis (Fig. 1) but changing the temperature did, as is illustrated by the Arrhenius plot of the data in Fig. 2. A discontinuity in the curve occurred at 302°K (29°C); at lower temperatures almost no hemolysis occurred.

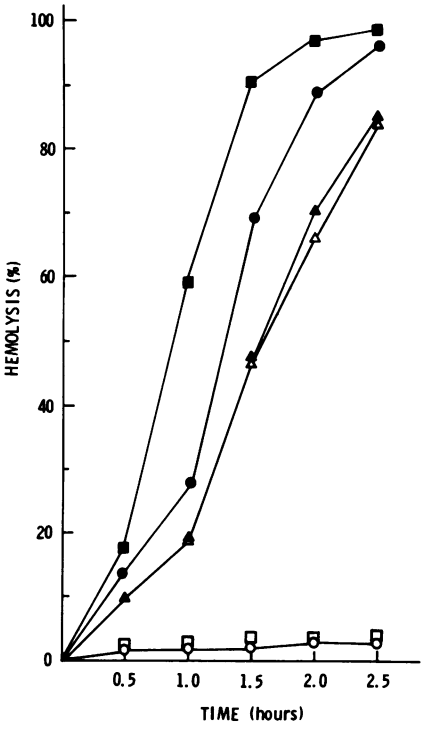

FIGURE 1 Time-course of FP-induced hemolysis. Aliquots of a 0.5% suspension of washed erythrocytes were incubated in the standard medium with 2 μM (□), 5 μM (▲), 6 μM (●), 7 μM (■), or no FP (○) for the indicated time intervals under room air at 37°C and pH 7.4 with exposure to room light. Incubation under the same conditions with 5 μM FP in the dark (△).

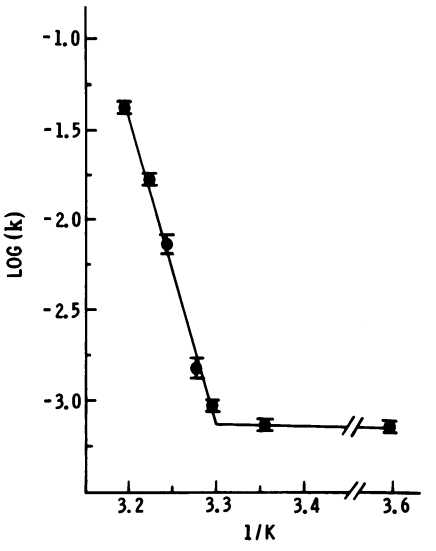

FIGURE 2 Arrhenius plot of the rapid phase of FP-induced hemolysis. Suspensions of washed erythrocytes were incubated for various lengths of time with 5 μM FP under the conditions described for Fig. 1 except that the incubation temperature was varied in separate experiments between 4 and 40°C. The ordinate shows the rate constants (k) for the rapid phase of hemolysis (between 90 and 150 min of incubation), that were estimated from semilogarithmic graphs of the percentage of nonhemolyzed erythrocytes remaining at the various time intervals. Each point shows the mean±SE for three experiments. 1/K shown on the abscissa is equal to 10^3/absolute temperature.

In the initial phase of damage from FP, there was a massive loss of potassium (Fig. 3). This loss reached 90% of the total present in the erythrocytes within 30 min of incubation with 5 μM FP. The loss ap-

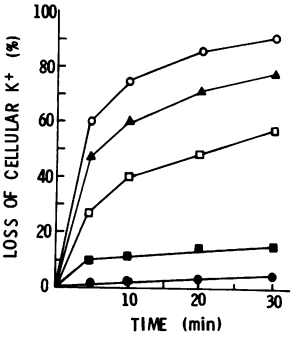

FIGURE 3 Effect of FP on potassium loss from erythrocytes. Aliquots of a 0.5% suspension of washed erythrocytes were incubated under the conditions described for Fig. 1 for the indicated time intervals with 1 μM(■), 2 μM (□), 3 μM (▲), 5 μM (○), or no FP (●). Potassium loss was measured as described in the text.

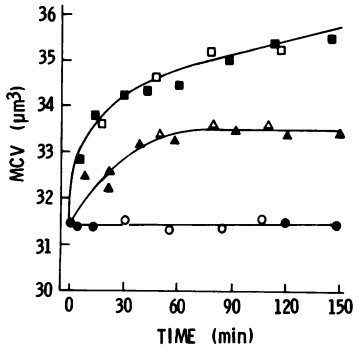

FIGURE 4 Effect of FP on erythrocyte swelling. In two separate experiments, aliquots of a 0.5% suspension of erythrocytes were incubated under the conditions described for Fig. 1 with 3 μM (△, ▲), 5 μM (□, ■), or no FP (○, ●). At the indicated time intervals, 10-μl samples of the suspensions were diluted to 20 ml with the standard medium at 37°C, and the mean cell volume (MCV) was measured without delay using a Coulter Channelyzer, model H_4.

proached its maximum within 5 min and was completed during the lag phase. Even low concentrations of FP (1 or 2 μM), caused significant potassium loss. In preliminary experiments, conducting the incubation under nitrogen had no effect on potassium loss (data not shown). Moreover, potassium loss was the same at 25°C as at 37°C, which is in contrast to the effect of temperature on hemolysis. The erythrocytes also swelled (Fig. 4), became spherocytic by microscopic examination, and exhibited increased osmotic fragility early in the course of exposure to FP (Fig. 5). The swelling occurred coincidentally with potassium loss and preceded hemolysis. The increased susceptibility to hypotonic lysis could be detected with FP concentrations as low as 0.5 μM (Fig. 5).

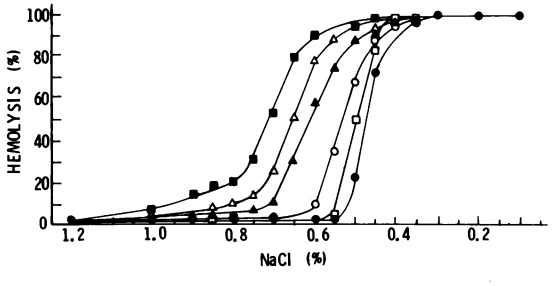

FIGURE 5 Effect of FP on the osmotic fragility of erythrocytes. Aliquots of a 0.5% suspension of erythrocytes were preincubated under the conditions described for Fig. 1 for 15 min with 0.5 μM (□), 1 μM (○), 1.5 μM (▲), 2 μM (△), 3 μM (■), or no FP (●). After preincubation the erythrocytes were collected by centrifugation at 300 *g* for 5 min and gently resuspended in the standard medium at room temperature to achieve an hematocrit of 30%. This suspension was used for determination of osmotic fragility by the method of Dacie and Lewis (13).

Continuing the study of osmotic effects in the hemolytic response to FP, sucrose was added to the suspending medium after various periods of incubation with FP. In a concentration of 0.1 M, sucrose completely stopped hemolysis (Fig. 6). The inset of Fig. 6 shows the concentration dependence of the effect of sucrose. There was a progressive decrease in hemolysis as the concentration was increased with almost complete protection being afforded by 0.05 M sucrose. In complementary experiments, sucrose was found to have no effect on the potassium loss induced by FP.

The effect of FP on erythrocyte ATP concentrations is shown in Fig. 7. The concentration of ATP did not change during the first 10 min of exposure to 2.5 or 5 μM FP. Thereafter, ATP concentrations decreased significantly. Thus, potassium loss (Fig. 3) preceded ATP depletion which in turn preceded the rapid phase of hemolysis (Fig. 1). Erythrocyte glutathione concentrations also were measured during incubation with 5 μM FP under the conditions described in the legend

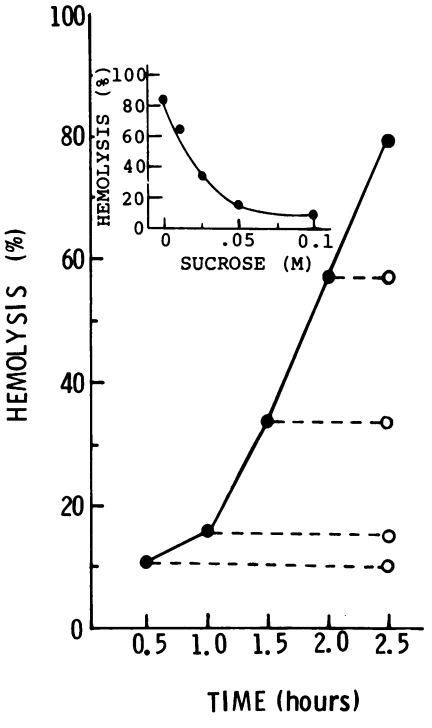

FIGURE 6 Inhibitory effect of sucrose on FP-induced hemolysis. Aliquots of a 0.5% suspension of washed erythrocytes were incubated with 5 μM FP under the conditions described for Fig. 1. At the indicated time intervals, sucrose was added to achieve a final concentration of 0.1 M. Immediately thereafter, 1 ml of the suspension was withdrawn for determination of the extent of hemolysis; incubation of the remaining suspension was continued to complete a total period of 2.5 h. Hemolysis at the time of addition of sucrose (●); hemolysis at the end of incubation (○). The inset shows the concentration dependence of the effect of sucrose added at the beginning of incubation.

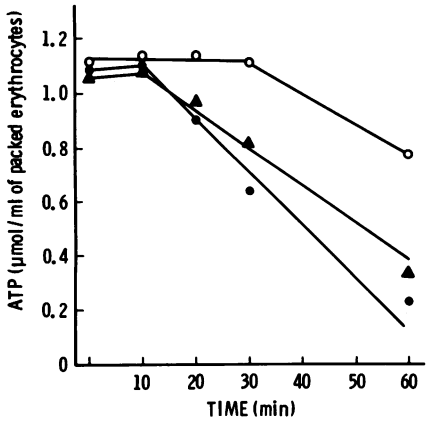

FIGURE 7 Effect of FP on ATP concentrations in erythrocytes. Aliquots of a 0.5% suspension of washed erythrocytes were incubated under the conditions described in Fig. 1 for the indicated time intervals with 2.5 μM (▲), 5 μM (●), or no FP (○). To determine the concentration of ATP, the erythrocytes were collected by centrifugation and extracted with cold 0.4 M perchloric acid. The perchloric acid extract was neutralized with cold 5 M K_2CO_3, the resulting potassium perchlorate was removed by centrifugation, and ATP was measured in the supernatant fluid by the firefly luciferase method (14).

for Fig. 1. In comparison with erythrocytes treated similarly except for no exposure to FP, the glutathione concentrations of FP-treated erythrocytes decreased only 11% during a 30-min period of incubation.

Because of the possibility that FP could damage the erythrocyte membrane by stimulating the production of free radicals or otherwise by serving as a prooxidant, membrane lipid peroxidation was evaluated (Table I). No increase in malonyldialdehyde was detected after treating erythrocytes with FP. Malonyldialdehyde was readily detected, however, in control experiments in which hydrogen peroxide was used to induce hemolysis. In related studies, various free radical scavengers were found to have no effect on FP-induced hemolysis (Table II). The only compounds that protected against hemolysis by FP were cysteine, dithiothreitol, and mercaptoethanol (Table II). Dithiothreitol and mercaptoethanol also were found to cause a shift in the absorption spectrum of FP. In the standard medium at pH 7.4, the absorption spectrum of FP had a broad peak between 360 and 385 nm. Upon addition of the sulfhydryl compounds the spectrum shifted, exhibiting a shoulder beginning at 385 nm and a peak at 405 nm. This shift reveals that the sulfhydryl compounds can interact directly with FP.

DISCUSSION

These results indicate that FP causes hemolysis by a colloid-osmotic mechanism. The sequence of events leading to hemolysis may be reconstructed as follows. FP impairs the ability of the erythrocyte membrane to maintain ion gradients, potassium leaks out (Fig. 3), water enters due to the osmotic gradient created by hemoglobin and other molecules trapped inside, the erythrocyte swells (Fig. 4), further damage occurs, and finally hemoglobin is lost (Fig. 1). As expected

TABLE I
Effect of FP on Hemolysis and Lipid Peroxidation in Normal Mouse Erythrocytes

Compound	Test	Time					
		0	30	60	90	120	150
		min					
None*	Thiobarbituric acid test (A_{534} nm)	0.013	0.014	0.016	0.012	0.013	0.016
	Hemolysis, %	—	—	2	—	—	3
FP (8 μM)*	Thiobarbituric acid test (A_{534} nm)	0.012	0.013	0.014	0.012	0.015	0.017
	Hemolysis, %	—	—	16	—	—	51
FP (16 μM)*	Thiobarbituric acid test (A_{534} nm)	0.011	0.013	0.016	0.011	0.014	0.016
	Hemolysis, %	—	—	62	—	—	96
H_2O_2‡	Thiobarbituric acid test (A_{534} nm)	—	—	0.087	—	—	0.107
	Hemolysis, %	—	—	36	—	—	86

* 2% suspensions of washed, normal mouse erythrocytes were incubated with or without the indicated concentrations of FP at 37°C for 2.5 h in a metabolic shaker as described in Fig. 1. Lipid peroxidation was assessed by measuring the formation of malonyldialdehyde colorimetrically using the thiobarbituric acid test as described in the text; light absorption at 534 nm is shown. The extent of hemolysis was measured as described in the text.

‡ A 2% suspension of washed, normal mouse erythrocytes was preincubated at 37°C and pH 7.4 for 1 h. Cells were spun down and resuspended at a concentration of 2% in fresh medium before the addition of sufficient H_2O_2 to achieve a concentration of 0.25% for incubation at 37°C and pH 7.4 for the indicated time intervals.

TABLE II
*Effect of Free Radical Scavengers and Sulfhydryl Compounds on FP-Induced Hemolysis**

Compound added	Concentration	Relative hemolysis
FP alone	—	100%
Benzoate	3.3 mM	95%
Dimethyl sulfoxide	3.3%	97%
EDTA	5 mM	91%
Formate	5 mM	100%
Histidine	5 mM	99%
Hypoxanthine	3 mM	110%
Mannitol	5 mM	105%
Cysteine	5 mM	18%
Dithiothreitol	5 mM	8%
Mercaptoethanol	5 mM	13%

* Suspensions of washed, normal mouse erythrocytes (0.5–0.75%) were incubated at 37°C and pH 7.4 for 2.5 h with 5 μM FP with or without the addition of one of the indicated compounds. At the end of the incubation, hemolysis was measured as described in the text. The extent of hemolysis of erythrocytes incubated with FP alone was taken as 100% for calculation of relative hemolysis. The values shown are representative of three or more experiments with each compound except mercaptoethanol, which was studied only once.

with this mechanism of hemolysis, erythrocytes treated with FP are extraordinarily susceptible to lysis by hypotonic solutions (Fig. 5), and they are protected from hemolysis when sucrose is added to the suspending medium (Fig. 6). Several years ago, Jacob and associates summarized similar evidence that a colloid-osmotic mechanism contributes to the destruction of erythrocytes treated with sulfhydryl blocking agents (17) and of erythrocytes harboring heme-depleted Heinz bodies (3). In addition to sucrose, Jacob and associates (17) found that albumin protects erythrocytes from hemolysis by sulfhydryl blocking agents. We did not include albumin in our studies because it is known to bind FP and remove it from the erythrocyte surface (10).

The hemolytic process induced by FP has at least two phases. In the first phase, there is little or no loss of hemoglobin from the erythrocyte, but there is a massive loss of potassium. The potassium loss precedes the depletion of glutathione and ATP, and it is not inhibited by reducing the incubation temperature from 37 to 25°C or by adding sucrose to the suspending medium. In the second phase, massive loss of hemoglobin occurs; this phase is inhibited by reducing the incubation temperature from 37 to 29°C and by the presence of sucrose. The biochemical lesions responsible for the potassium loss and hemolysis have not been identified; but no evidence of peroxidation of membrane lipids was found, and the lesions were not prevented by the presence of free radical scavengers or by incubation in the dark. A possible lead is the observation that sulfhydryl compounds protect the erythrocyte from FP (Table II). This protective effect deserves further study. It could be due to an interaction of the sulfhydryl compounds directly with FP, to an interaction with a component of the erythrocyte, or both.

Despite our incomplete understanding of the biochemical lesions induced by FP, it is appropriate to ask whether FP liberated intracellularly from abnormal hemoglobins or as a result of oxidant stress would function as a hemolytic agent. In the present work, we did not study the accumulation of FP intracellularly, but evidence has been obtained recently that FP can penetrate into mouse erythroleukemia cells (18). The possibility that intracellular FP functions as a hemolytic agent is supported by the observations of Goldberg and Stern, who found that the ghosts of erythrocytes lysed by the oxidants, 1,4-naphthoquinone-2-sulfonate or dihydroxyfumaric acid, contain a pigment (19, 20) with spectral characteristics similar to those of FP bound to spectrin (21). They propose that this unidentified pigment is derived from the heme moiety of hemoglobin (19, 20) and that it mediates the hemolytic effect of oxidants (19, 20). They found no lipid peroxidation or oxidation of membrane sulfhydryl groups in their experiments (19, 20). Other evidence supporting intracellular FP as a hemolytic agent is the fact that oxidation of intracellular hemoglobin results in hemolysis only under conditions that would permit the release of FP (22). For example, nitrites cause methemoglobinemia but not FP release (23) or hemolysis (24–26); carbon monoxide prevents FP release (3, 27) and also prevents hemolysis from oxidants (20); and easily oxidized abnormal hemoglobins that do not lead to the production of heme-depleted Heinz bodies are not associated with hemolytic anemia (22). These observations and our results justify further evaluation of the hypothesis that intracellular FP functions as a hemolytic agent.

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