Comparative Study of Protoporphyrins in Erythropoietic Protoporphyria and Griseofulvin-Induced Murine Protoporphyria

BINDING AFFINITIES, DISTRIBUTION, AND FLUORESCENCE SPECTRA IN VARIOUS BLOOD FRACTIONS

MAUREEN B. POH-FITZPATRICK and ANGELO A. LAMOLA with the assistance of GREGORY L. ZALAR, MARK WEINSTEIN, FRANK DOLEIDEN, and MARIA FREEMAN

From the Department of Dermatology, Columbia University College of Physicians and Surgeons, New York 10032, and the Bell Telephone Laboratories, Murray Hill, New Jersey 07974

ABSTRACT Excess erythrocyte protoporphyrins of human congenital erythropoietic protoporphyria and of griseofulvin-induced murine hepatic protoporphyria were found to be associated with hemoglobin and stroma fractions in similar relationships. More than 99.5% of total erythrocyte protoporphyrin was bound to hemoglobin in each case. However, profound differences were found when protoporphyrin concentration was measured in erythrocytes that had been segregated into populations of progressive age on discontinuous density gradients. In erythropoietic protoporphyria, porphyrin content diminished rapidly with age; in murine protoporphyria, the aging erythrocyte populations became progressively more porphyrin rich. In vitro diffusion of protoporphyrin from plasma across the intact erythrocyte membrane was demonstrated. The equimolar binding affinity of protoporphyrin to hemoglobin was shown to be 40 times that of protoporphyrin to serum albumin. This strong affinity provides the driving force for the observed transmembrane diffusion, and explains the high erythrocyte/plasma porphyrin ratio in murine hepatic protoporphyria. The opposite rapid efflux of intraerythrocytic protoporphyrin into plasma previously shown in uncomplicated erythropoietic protoporphyria occurs despite this strong hemoglobin affinity, implying continuous efficient clearance of protoporphyrin from plasma by the liver. Furthermore, these and other data suggest that a hepatic synthetic source for any significant fraction of the blood protoporphyrin in erythropoietic protoporphyria is highly improbable.

INTRODUCTION

Congenital protoporphyria in humans was first named erythropoietic protoporphyria (EPP)[1] (1–3) based upon the large amounts of protoporphyrin IX (PP) found in the blood of patients with the disease which was thought to be synthesized in the bone marrow. However, the observation was made that the daily excretion of PP in the feces of these patients approached the total excess PP in the circulating erythrocyte mass. It was clear that this amount could not be derived from senescent erythrocytes only (4). Some investigators proposed the liver as an important additional source of PP in this disease. The occurrence of PP crystals in the liver accompanied by hepatic dysfunction in some EPP patients lent support to this hypothesis (5, 6). Others suggested that the excretory load of PP measurable in EPP reflected an extremely rapid release of PP from maturing erythrocytes (7). Recently, two of us and others have published evidence that strongly supports the erythropoietic system as the predominant, if not the sole, source of excess PP in EPP (8). It was confirmed that a rapid

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[1] *Abbreviations used in this paper*: DDAO, dodecyldimethylamine oxide; EPP, erythropoietic protoporphyria (-ric); GF, griseofulvin; GFPP, griseofulvin-induced (murine) protoporphyria (-ric); HSA, human serum albumin; PBS, phosphate buffered saline; PP, protoporphyrin; ZnPP, zinc protoporphyrin.

leak of PP from erythrocytes in EPP does indeed occur in vivo, sufficient to account for the entire excess of PP in the feces. Hepatic disorders associated with EPP are probably consequences of a constantly enormous load (~50 mg/day) of PP effluxed into the plasma from erythrocytes which must be cleared by the liver.

Acquired protoporphyria is extremely rare in man, but protoporphyria can be easily induced in mice with certain drugs, among which is griseofulvin (GF) (9–11). Murine protoporphyria induced by oral administration of GF has been used as an experimental model for human EPP (12, 13). The site of excess PP synthesis in GF-induced murine protoporphyria (GFPP) is thought to be primarily, if not solely, hepatic (14). However, the concentration of PP associated with the circulating erythrocytes in GFPP can become comparable to that observed in EPP (9, 13, 14).

We have developed spectrofluorometric techniques which give information about the binding sites of PP associated with erythrocytes (15, 16). We have also developed criteria, based on the distribution of PP among circulating erythrocytes of different ages, for the determination of rate and direction of flux of PP in the blood pool (8). We have applied these methods to the question of the origin of erythrocyte PP in GFPP. We performed this study for two reasons: (*a*) to assess more fully GFPP as a model for EPP and, (*b*) to assess more fully tissue localization of PP as a criterion for tissue source of PP.

METHODS

Induction of porphyria in mice and blood sampling. 8-wk-old female Swiss-Webster albino mice were rendered protoporphyric by feeding ad libitum with powdered feed containing 2% micronized GF (Ayerst Laboratories, New York). Groups of three experimental mice and one control (unadulterated feed) mouse were sacrificed at intervals from 1 day to 7 wk after commencement of GF administration. Mice anesthetized with ether were exsanguinated by intracardiac puncture into a heparinized syringe. 1–2 ml of blood could be obtained from each mouse. Plasma and erythrocytes were immediately separated by centrifugation (5,000 rpm × 10 min) to minimize hemolytic contamination of plasma with hemoglobin. Buffy coats were removed. Blood was obtained from patients with EPP by venipuncture.

Intoxication of mice with lead. Swiss-Webster albino mice were acutely poisoned with lead by adding the metal to their drinking water at 10 or 20 g Pb/liter as the acetate salt.

Fractionation of erythrocytes by density gradient centrifugation. Erythrocytes from individual control mice, GFPP mice, or lead-intoxicated mice were fractionated by ultracentrifugation on discontinuous density gradients of arabinogalactan (Stractan II, St. Regis Paper Co., New York) as described by Corash et al. (17). After removal of plasma and the buffy coat from a blood sample, the erythrocytes were washed with the phosphate-buffered glucose solution described by Corash et al. About 0.5 ml of washed packed cells was transferred onto a gradient prepared as described except that the densities of the Stractan layers were 1.090, 1.087, 1.084, and 1.081 to accommodate lower densities of murine erythrocytes compared to human erythrocytes. Five fractions were obtained after centrifugation. The fractions were carefully separated, the volume of each fraction and its hemoglobin concentration were determined, and the various other analyses were performed. Fractionations of erythrocytes from patients with EPP were performed at Stractan layer densities of 1.097, 1.093, 1.089, and 1.085.

Preparation of blood fractions. Hemolysates and hemoglobin-free stroma were prepared from erythrocytes by addition of 20 vol or more of 25–30-mosmol phosphate-buffered saline, pH 7.9, to the cells. The stroma were pelleted at 35,000 *g* for 20 min at 4°C in a Sorvall RC2-B centrifuge (Du Pont Co., Instrument Products Div., Sorvall Operations, Wilmington, Del.). The supernatant hemolysate was removed and saved. The pellet was resuspended and washed three times with 25 vol of the same hypotonic buffer solution. The resultant white stroma contained ≤.03 mg hemoglobin per milligram protein. Hemoglobin content of stroma and of plasma was determined by the orthotolidine method of Lewis (18). Protein content of stroma was determined according to Sutherland (19). Hemoglobin concentrations of hemolysates were assayed according to Van Kampen and Zijlstra (20).

Purified human hemoglobin was prepared from a hemolysate by fractionation on a Sephadex G-100 column (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) as previously described (16).

Human serum albumin (HSA) was prepared from pooled plasma by separation on a Sephadex G-150 column by the method of Flodin and Killander (21). The pooled fractions from the third band eluted, which contains the HSA, were dialyzed against 0.1 M Tris-HCl, 0.05 M NaCl, pH 8.4, placed on a DEAE-cellulose (DES-52) column and eluted with a salt gradient from 0.05 to 0.2 M NaCl. The HSA eluted in the second band at about 0.15 M NaCl. After dialysis against 0.01 M Tris-HCl, 0.9% NaCl, pH 7.4, the solution was concentrated using an Amicon Minicon concentrator (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) until the concentration of HSA was about 0.5 mM as judged by its absorbance at 280 nm, using E(mM) = 36.

Spectrofluorometric analyses. Fluorescence excitation and emission spectra were obtained for intact erythrocytes, plasma, and hemolysates of both murine and human specimens diluted in isotonic phosphate-buffered saline, pH 7.4, (PBS), and in PBS containing 2% dodecyldimethylamine oxide (DDAO), a nonionic detergent, as described by Poh-Fitzpatrick and Lamola (15). Spectra were obtained from stroma suspended in PBS to concentrations of approximately 0.1–0.5 mg protein per milliliter. All spectra were recorded using a Hitachi-Perkin Elmer MPF-3L spectrofluorometer (The Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) equipped with a Hamamatsu R446UR (red sensitive) photomultiplier (Hamamatsu Corporation, Middlesex, N. J.).

Preparation of porphyrin-hemoglobin and porphyrin-stroma complexes. Protoporphyrin IX (Porphyrin Products, Logan, Utah) was dissolved in a minimal amount of 8 N HCl, then diluted with PBS (pH 7.4) to a concentration approximating 1 μg/ml. Hemoglobin-free stroma (0.5–1 ml) prepared from normal mouse and human erythrocytes as described above were separately incubated with 5 ml of PP-buffer solution for 5 min, at 25°C, in the dark. The PP-containing stroma were pelleted and washed three times with PBS. Final pellets were suspended in 5 ml PBS.

Normal mouse and human hemolysates were incubated with equal volumes of PP-buffer solution for 5 min in the dark to form the PP-hemoglobin complexes.

Fluorescence microscopy. A Leitz Ortholux fluorescence microscope (Leitz/Opto-Metric Industrial Div. of E. Leitz Inc., Rockleigh, N. J.) equipped with an epilumination system and a mercury arc was used. The 405-nm emission of the lamp was isolated by appropriate primary filtration. Fluorescence was observed through a 530-mm secondary filter.

Porphyrin assays. 20 μl of each well-mixed specimen of packed erythrocytes was assayed for protoporphyrin as previously described (22, 23). An aliquot of each plasma specimen was similarly assayed. PP content of hemolysates and stroma was measured by a modification of the same rapid fluorometric microassay. Porphyrin values in plasma were corrected by subtraction of PP content due to hemolysis.

RESULTS

Levels, distribution, and fluorescence of PP in GFPP mouse blood. Concentrations of PP in plasma and erythrocytes were measured in blood samples from mice sacrificed daily for the 1st 4 days after commencement of GF feeding and then at weekly intervals. Blood samples from control mice were examined in parallel. Data are given in Table I.

Concentrations of PP in plasma and erythrocytes of mice fed griseofulvin for 1 week rose to levels comparable with those found in EPP. Base-line values remained unchanged for the control mice. Plasma PP in experimental mice was noted to rise rapidly during the 1st 2 days after the start of GF feeding, while erythrocyte PP became noticeably elevated only after about 3 days.

TABLE I
PP in Erythrocytes and Plasma

Specimen	Mean PP concentration	No. of specimens	Range	SE
	μg/dl			
Erythrocytes				
Human				
Normal	24	60	5–67	1.7
EPP	954	21	349–1,850	99.1
Murine				
Normal	54	15	35–100	4.2
GFPP				
Day 1	59	10	26–96	7.8
2	57	7	30–92	7.8
3	72	6	42–110	9.6
4	135	4	72–275	32.4
Wk 1	938	6	445–1,550	170.0
4	2,000	3	1,500–2,600	262.5
7	2,280	2	1,927–2,633	249.6
Plasma				
Human				
Normal	0.2	60	0–0.9	0.02
EPP	26.8	21	6.8–125	5.8
Murine				
Normal	3.0	14	0.6–7.6	0.4
GFPP				
Day 1	4.6	10	1.6–7.8	0.6
2	8.2	7	5.2–13.2	1.1
3	18.6	6	7.4–27.0	3.4
4	38.8	5	15.0–93.0	12.6
Wk 1	80.3	6	42–120.6	12.1
4	112.3	3	75–180	27.7
7	310.0	2	300–320	7.1

As found for stroma from EPP patients, stroma from GFPP mice contained only a small fraction, about 0.5%, of the total erythrocyte PP. The majority of the PP is associated with the cytosol hemoglobin in both cases.

The fluorescence signal for GFPP murine specimens increased in magnitude as the PP levels rose above normal levels, but its excitation and emission remained unchanged. Control mouse erythrocytes and hemolysates had emission spectra which peaked at 595 nm. A comparable fluorescence observed in similar normal human preparations is characteristic for zinc-protoporphyrin hemoglobin complexes. However, control mice erythrocytes showed metal-free PP emission as well.

Characteristic fluorescence excitation and emission maxima obtained in these studies are listed in Table II for all of the preparations described. Spectra for GFPP erythrocytes and plasma are shown in Fig. 1.

Levels, distribution, and fluorescence of porphyrins in lead-poisoned mouse blood. While plasma levels remained unchanged, levels of acid-extractable PP rose to two to three times normal in the erythrocytes of mice with lead in their drinking water. Fluorescence spectra of the erythrocytes of these mice revealed mixtures of zinc protoporphyrin (ZnPP) and PP. The ratio of ZnPP to PP was variable, but generally was near unity.

Analyses of erythrocyte fractions of different mean densities. Reticulocyte counts, performed in the standard manner, were obtained for each of the fractions from the density gradients. For all GFPP, lead-poisoned, and control mice, reticulocyte percentage decreased as mean density increased for the four suspended layers. The count in the fifth or most dense layer, found at the bottom of the tube, was usually comparable to that in the least dense layer. Data are shown in Fig. 2.

The acid-extractable PP level in lead-poisoned mouse erythrocytes decreased as mean density increased for the four suspended layers. Like the reticulocyte count, the PP level rose again in the most dense layer. This pattern was found consistently for erythrocyte samples from mice poisoned with lead for from 1 wk to 4 wk. Representative data are shown in Fig. 2. Spectral analysis of the fluorescence from the fractions of different densities for a mouse fed lead for 2 wk revealed that the ratio of ZnPP to PP was about 1.5 in the four suspended layers and about 0.75 in the fifth (most dense) layer.

An inverse relationship between PP levels and the densities of the fractions was found for erythrocytes

TABLE II

Characteristic Maxima of Spectra Recorded for Human and Murine Blood Fractions by Direct Spectrometry

Specimen	Diluent	Emission	Excitation
		nm	
Erythrocytes			
Human			
Normal	Phosphate-buffered saline (PBS)	595 (weak)	425
	PBS with 2% dodecyldimethylamine oxide (PBS-DDAO)	595 (weak)	425
EPP	PBS	625	397
	PBS—DDAO	634	408
Murine			
Normal	PBS	595 (weak)	—
	PBS—DDAO	—	—
GFPP	PBS	628	397
	PBS—DDAO	634	412
Lead-poisoned	PBS	595, 628	427, 399
	PBS—DDAO	595, 634	428, 398
Plasma			
Human			
Normal	PBS, PBS—DDAO	—	—
EPP	PBS, PBS—DDAO	634	408
Murine			
Normal	PBS, PBS—DDAO	—	—
GFPP	PBS, PBS—DDAO	634	—
Hemolysate			
Human			
Normal	PBS, PBS—DDAO	595 (weak)	425
EPP	PBS	625	397
	PBS—DDAO	634	408
Murine			
Normal	PBS, PBS—DDAO	595 (weak)	—
GFPP	PBS	628	395
Normal with added protoporphyrin	PBS	637	—
		628	—
Stroma			
Human			
Normal	PBS	620 (very weak)	—
EPP	PBS	634	—
	PBS—DDAO	634	near 412
Murine			
Normal	PBS	—	—
GFPP	PBS—	634	—
	PBS—DDAO	635	near 412

from GFPP mice; PP concentration rose with increasing mean density in the four suspended layers and fell in the most dense layer. This pattern was found consistently in erythrocyte samples from mice fed GF for 1–7 wk. A marked increase in the slope of the curve of PP vs. cell density (first four fractions) as a function of duration of GF administration is evident in Fig. 2.

Fluorescence microscopical observations. Peripheral blood smears obtained from patients known to have EPP showed visually observable fluorescence which varied in intensity from very strong to none among different erythrocytes, as previously described (24). Peripheral blood from GFPP mice, on the other hand, showed fluorescence from essentially all of the erythrocytes with varied intensities from strong to moderate. Similar preparations from lead-intoxicated mice exhibited weak fluorescence in many cells which faded too rapidly to permit close estimation of percentage fluorocytes. Rare fluorocytes were observed in blood or bone marrow smears from control mice.

Fluorescing cells were observed in marrow preparations from GFPP mice. Marrows of lead-poisoned mice all contained large numbers of fluorocytes. Precise

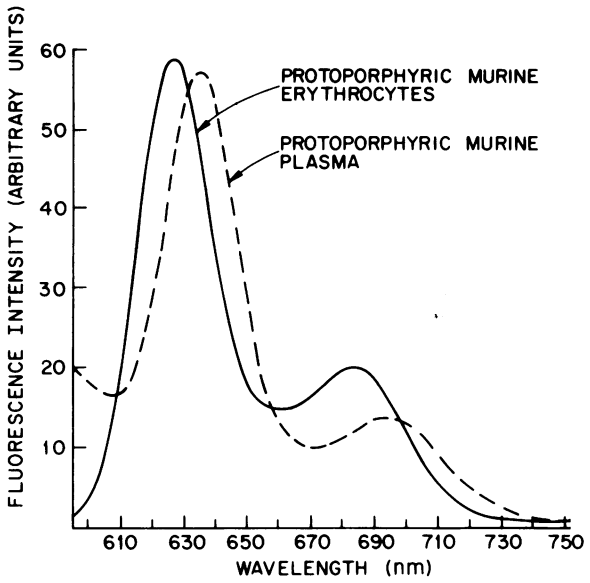

FIGURE 1 Fluorescence spectra of a 1:1,000 suspension of erythrocytes, and of a 1:4 dilution of plasma from GFPP mice. Excitation wavelengths were 400 and 408 nm, respectively.

numbers and stages of maturation of the fluorocytes could not be determined beyond identification as erythroid elements since fading, though less rapid than in the peripheral smears of lead-poisoned mice, was encountered.

Relative binding of PP to HSA, human hemoglobin, and human erythrocyte stroma. Mixtures of hemoglobin at 1 μM and HSA from 1 to 400 μM were prepared in PBS. To 2-ml aliquots of these mixtures was added 0.2 ml of a 2-μM solution of PP in PBS. The sealed sample tubes were shaken gently for 6–24 h in a water bath at 25°C in the dark. Fluorescence spectra of the incubated mixtures were recorded at excitation wavelength 405 nm, excitation slit width 20 nm, and emission slit width 8 nm. Spectra and relative fluorescence intensities from mixtures of PP (0.2 μM) and hemoglobin (1 μM), and PP (0.2 μM) and HSA (10 μM) were also obtained under the same conditions. Fluorescence spectra of the various mixtures of PP, hemoglobin, and HSA did not change for incubation times between 6 and 24 h. It was concluded that spectra recorded for samples incubated for 24 h represented equilibrated distributions of PP. For HSA-hemoglobin ratios of 5 or less, the fluorescence spectrum of the mixture was virtually identical to that of the pure hemoglobin-PP complex, i.e. maximum at 625 nm (Fig. 3). For HSA/hemoglobin ratios of 200 or greater, the spectrum was virtually identical to that of the pure HSA-PP complex, i.e. maximum at 634 nm. For HSA/hemoglobin ratios between 10 and 100, the spectra clearly indicated mixtures of comparable amounts of the two complexes.

The spectrum observed for a HSA/hemoglobin ratio of 40 is shown in Fig. 3. This spectrum is close to that expected for a 1:1 mixture of hemoglobin-PP and HSA-PP. Thus, the ratio of binding constants for hemoglobin-PP and HSA-PP is about 40. The value determined from several measurements is 45±10.

That the PP binding equilibrium for murine albumin and hemoglobin also lies strongly on the hemoglobin side was shown by the following experiment. To 0.25 ml of murine plasma containing about 300 μg/dl (~5 μM) PP, was added 0.1 ml of the homologous hemolysate which contained about 1.3 g/dl (~200 μM) hemoglobin. After standing for 6 h at room temperature, the mixture was examined spectrofluorometrically. The PP fluorescence observed was characteristic of hemoglobin-PP.

Mixtures of hemoglobin at 1 μM and human erythrocyte stroma from 0.06 to 1.0 mg protein/ml were prepared in PBS. To 2-ml aliquots of these mixtures, was added 0.2 ml of a 2 μM solution of PP in PBS. After incubation at 25°C for 12–24 h, the mixtures were analyzed fluorometrically as described for the hemoglobin-HSA mixtures.

The fluorescence spectrum of PP bound to stroma

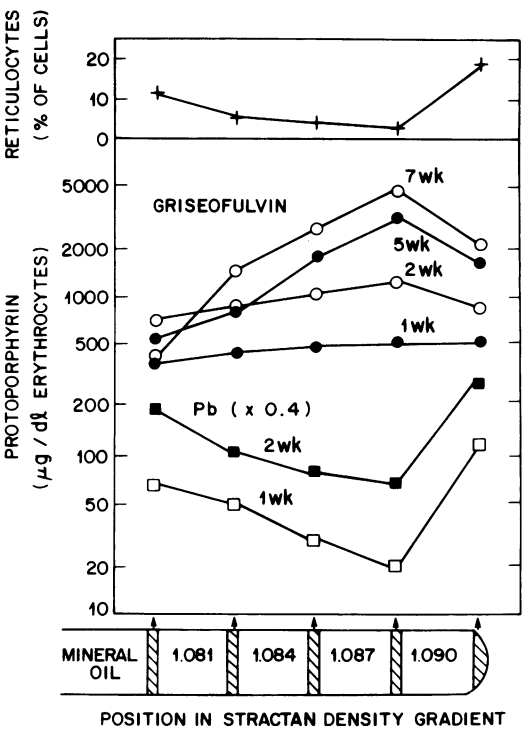

FIGURE 2 Typical values of reticulocyte counts (+) and erythrocyte protoporphyrin for GFPP (○, ●) and lead-intoxicated (□, ■) mice, in erythrocyte fractions of increasing mean density. Below the ordinate is represented a discontinuous density gradient, with hatched areas indicating the erythrocyte layers. Data for mice fed GF for 1–7 wk, and for mice intoxicated with lead for 1 and 2 wk are shown.

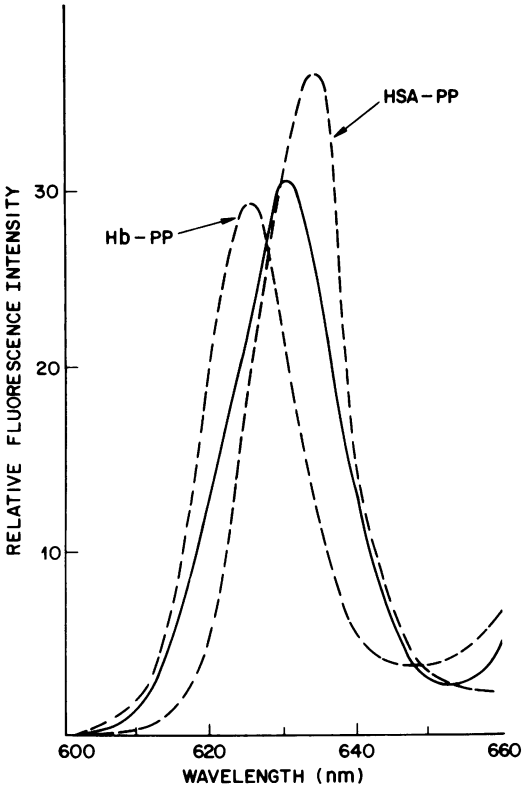

FIGURE 3 Fluorescence spectra of mixtures of PP (0.1 μM in all cases) with human hemoglobin (Hb-PP) and with human serum albumin (HSA-PP). The solid curve shows the spectrum of a mixture of PP with hemoglobin and albumin in the ratio 1:40. The protein concentrations were at least 1 μM.

is very similar to that of the HSA-PP complex, i.e. maximum at 634 nm. Thus, the same kind of fluorometric analysis as used for the hemoglobin-HSA mixtures, was used to determine the relative binding strength of PP to human hemoglobin and stroma. The samples containing 1 μM PP and stroma corresponding to about 0.28 mg protein per milliliter were found to give equal proportions of hemoglobin-PP and stroma-PP fluorescence. Taking 600 mg for the weight of stroma protein in 100 ml of erythrocytes (25), the relative binding constant of PP to cytosol hemoglobin and to the erythrocyte membrane is calculated to be about 240. This value is in excellent agreement with the finding that in EPP <0.5% of the total erythrocyte PP is found in the stroma (16).

Fresh erythrocytes from an EPP patient were separated into five density fractions, and the PP concentrations in both the whole erythrocytes and stroma were measured for each fraction. The data (Table III) show that the ratio of cytosol PP to stroma PP is nearly the same for every fraction within the experimental error (about 10%).

Transfer of PP from human plasma to human erythrocytes. Fresh normal human blood was separated by centrifugation into cells and plasma, but the erythrocytes were not subsequently washed. To 10 vol of the plasma was added 1 vol of a PBS solution containing 1,200 μg/dl (20 μM) PP. This mixture was incubated at room temperature for 1 h. 9 ml of the synthetic protoporphyirc plasma was added to 8 ml of packed erythrocytes in each of several 25-ml Erlenmeyer flasks and sealed. The flasks were gently shaken in a water bath at 37°C carefully protected from light. Samples were removed after 6, 24, and 30 h and analyzed as follows. The erythrocytes were separated from the plasma fraction by centrifugation. The cells were washed three times with PBS, and both the plasma fraction and washed cells were analyzed for PP by spectrofluorometry.

After 3 h no decrease in plasma PP or increase in erythrocyte PP outside of the error of the experiment was observed. After 6 h an increased erythrocyte level was observed, consistent with an observed 3–13% decrease in plasma PP. After 24 h 20–30% of the plasma PP was found associated with the erythrocytes. Fluorometric analysis indicated that most (>80%) of this erythrocyte PP was associated with the hemoglobin in the cells. At this time some hemoglobin was present in the plasma fraction due to the unavoidable small amount of hemolysis. Fluorometric analysis showed that the PP remaining in the plasma fraction was bound to this hemoglobin. Samples examined after 30 h of incubation showed a similar pattern.

DISCUSSION

On the basis of enzyme activities, Nakao et al. (14) concluded that the overproduction of PP in drug-induced mice occurs in hepatic, not erythroid, tissue. They suggested that the PP found in GFPP erythrocytes was taken up from plasma by the lipid-rich erythrocyte membranes. They speculated that similar uptake of PP by human EPP erythrocytes might also account for part of the total erythrocyte porphyrin found and implied a hepatic source for some of the

TABLE III
Density Fractionation of EPP Erythrocytes

Fraction	PP erythrocytes		Ratio
	Cytosol	Stroma	
	μg/dl		
1	4,630	12	380
2	2,450	8.4	290
3	1,820	6.5	285
4	1,300	4.1	315
5	1,080	2.9	380

erythrocyte PP in EPP. We have shown here that the bulk of the PP is associated with hemoglobin rather than membranes in GFPP murine erythrocytes. However, these observations do not disprove the speculations of Nakao et al., inasmuch as we found a very similar distribution of PP among blood components of patients with EPP.

The close agreement found in the distribution of PP among the plasma, erythrocyte stroma, and hemoglobin pools of the blood fails to help define any difference in sites of PP overproduction in GFPP and EPP. The small difference observed in the fluorescent spectral characteristics of the hemoglobin-PP complexes may reflect the amino acid sequence variations in the globin chains of mice and humans (26). Only the numbers of visually observable fluorocytes in the peripheral circulation (5–20% for EPP, 100% for GFPP) appear to differentiate between the two disorders.

Two conclusions might follow from the above observations: (*a*) The concept that the great majority of excess PP in EPP is synthesized in erythroid cells, or that the predominant PP source in GFPP is liver cells, is invalid. (*b*) Alternatively, average distribution of PP among blood component fractions is a poor criterion for assessment of the source of erythrocytic PP.

We tend to prefer the second conclusion because we had reported data which suggested that diffusion of intraerythrocytic PP into the plasma occurred at a rate sufficiently rapid to account for the amounts recovered from feces of patients with EPP without additional contribution from hepatic PP synthesis (8). We had confirmed nonuniform distribution of PP in erythrocytes of different ages in blood from patients with EPP. It was shown that the youngest cells contained very large concentrations of PP, which rapidly diminished to normal levels with progressive age. This rapid loss of erythrocyte PP explains the finding of only 5–20% fluorocytes in blood of EPP patients; the nonfluorescing cells are older and have lost their PP. The previously unreported observation of variation in fluorescence intensity among individual fluorocytes from GFPP mice suggested to us that nonuniform distribution of PP in different erythrocyte populations also occurs in GFPP, even though all erythrocytes are fluorescent.

To compare the distribution of PP in erythrocytes of different mean age from GFPP mice, washed murine erythrocytes were fractionated in the same system which had been successfully applied to human erythrocytes. Valid comparison depended on assurance that mouse erythrocytes would segregate into populations of progressive age when separated over the density gradients. Measurement of age-dependent enzymes was not used as the criterion of age in cell layers obtained from mouse blood inasmuch as the relationships of murine enzyme activities and cell age are not as well defined as in human erythrocytes. Reticulocyte counts were performed to approach some appreciation of the percentage of juvenile cells in the layers of progressive density. Although in a five-layer separation, the four suspended layers contained decreasing numbers of reticulocytes as expected in populations of increasing average ages, the fifth, and most dense, layer was consistently populated with numbers of reticulocytes which approached those of the topmost, and least dense, layers. Such inhomogeneity of reticulocytes in rodents have been observed by others. For example, Walter and Albertsson (27) identified two populations of reticulocytes in blood of phenylhydrazine-treated rats. These populations were associated with the opposite ends of the normal density distribution curve for rat erythrocytes similarly separated. We suspect a similar multiplicity in murine reticulocyte populations may explain our findings. However, it appeared necessary to confirm the progression of mature erythrocyte age with increasing density of the murine erythrocyte layers by other means. Lead interferes with heme synthesis such that ZnPP and metal-free PP accumulate in erythroid cells (8, 15, 16). Since this interference takes place only during the time hemoglobin is synthesized, only those erythrocytes produced after the start of lead administration should contain excess acid-extractable PP. Therefore, we turned to the procedure of lead poisoning a group of mice and separating their erythrocytes on identical gradients. Thus, it was possible to determine relative ages of erythrocyte layers in these bloods by measuring the erythrocyte protoporphyrin concentration in each layer. The results (Fig. 2) gave us confidence that separation by age of murine erythrocytes had been achieved for the upper four layers, and that the bottom layer contained numbers of very dense cells whose morphologic and biochemical characteristics were similar to the reticulocyte-rich topmost layers as well as the oldest erythrocytes.

Fig. 2 clearly shows increasing PP concentration in the four cell layers of progressive average age in blood of GFPP mice. The pronounced increase in the slope of the lines as the course of GF administration progressed from 1 to 7 wk is also of significance. Not only do the murine erythrocytes become more PP-laden with increasing age in circulation (in direct contrast with the rapid PP loss with age in EPP), but as the older erythrocytes are exposed to drug-induced plasma PP excesses for longer periods of time, they exhibit the capacity for progressively greater absorption of the PP. Thus, at 7 wk of GF ingestion, the oldest murine erythrocytes (murine erythrocyte life-span approximates 42 days) have been

bathed in PP-rich plasma for their entire life-spans. These cells have the greatest PP concentration among the GFPP murine blood preparations, and differ markedly from the youngest erythrocytes, the exposure of which to PP-rich plasma is short.

These results show that although the average partitioning of PP among blood components is virtually identical in GFPP mice and patients with EPP, the distributions of PP among erythrocytes of different ages are diametrically opposed. Our data is most consistent with a hepatic origin for the excess PP measured in plasma and erythrocytes of GFPP mice. At the same time, the data argue against the concept that significant amounts of the circulating-PP burden in EPP derive from hepatic tissue.

The plasma proteins that bind protoporphyrin most strongly appear to be hemopexin and albumin (28, 29). While it may be that hemopexin binds PP somewhat more strongly than does albumin, the large ratio of concentrations of albumin to hemopexin, approximately 100:1 in all species examined (30), should make albumin the main carrier of PP in plasma. Strong evidence that this is so is provided by spectrofluorometric data to be published separately.[2] Quantitative comparison of the binding strengths of PP to hemoglobin and to plasma albumin should, therefore, reflect the relative strengths of binding of PP to total plasma vs. hemoglobin. Because a relatively large amount of purified albumin was required, quantitative binding studies were performed only with HSA and human hemoglobin.

Direct competition experiments showed that PP binds about 40 times more strongly to human hemoglobin than it does to HSA in PBS. The molar ratio of hemoglobin to HSA in blood is about 6:1. Assuming that, at most, all other plasma sites together contribute to PP binding as importantly as HSA, one expects that at equilibrium, the ratio of PP bound to hemoglobin to PP bound to plasma proteins to be at least 120:1. That fluorescence characteristic of hemoglobin-PP was observed from a mixture of murine plasma and hemolysate is consistent with a binding constant ratio of this magnitude for the murine case as well. This large ratio can provide the driving force necessary to explain the observation of net diffusion of PP from plasma to intracellular hemoglobin in GFPP mice and the buildup of the observed high erythrocyte PP levels.

Competition experiments demonstrated that at equilibrium virtually all erythrocyte PP should be bound to the hemoglobin, and that only about 0.5% is expected to be localized in the membrane. Such large ratios of hemoglobin-PP to stroma-PP are observed for erythrocytes obtained from both EPP patients and GFPP mice. That the hemoglobin-PP and stroma-PP are in equilibrium in vivo is demonstrated by the invariance of their ratio for EPP erythrocytes of different age, of which the average total PP content varied by a factor of four (Table III). Diffusion of PP from plasma sites to sites on the cytosol hemoglobin of intact erythrocytes was demonstrated in vitro. The experiment is made difficult by the unavoidable hemolysis which occurs. The small amount of hemoglobin released into the plasma quickly scavenges PP. Fortunately, hemolysis occurred slowly enough under the experimental conditions to allow observation of buildup of hemoglobin-PP in intact erythrocytes incubated in PP-plasma. An estimated half-life for the process is on the order of 1 day at 37°C. The rate limiting step must be the transfer of PP from plasma (albumin) sites to the cell membrane. The rate of transfer from the membrane to internal hemoglobin appears to be somewhat faster. The transfer of PP from albumin to hemoglobin directly when both are in the same solution (no membrane) is faster yet; equilibrium is attained within 6 h. The kinetics of the transfer of plasma-PP to intracellular hemoglobin-PP in GFPP mice appear comparable to those in the in vitro experiment using human blood fractions in that plasma-PP was observed to rise for about 3 days in the mice before erythrocyte PP levels began to rise.

Observation of a high preference of PP to bind to hemoglobin rather than to sites in plasma explains the difficulty connected with demonstration of diffusion of PP out of EPP erythrocytes in vitro (8, 31). In experiments previously reported, EPP erythrocytes were incubated with 5 vol of PP-free ABO-compatible plasma. Under these conditions, not more than 5–10% of the total PP is expected to be present in the plasma fraction at equilibrium.

The strong preference of PP to bind to hemoglobin rather than to plasma sites has implications with reference to the rapid diffusion of PP from erythrocytes of EPP patients in vivo. For the observed rapid and continuous flow of PP from erythrocytes to occur, the plasma must be very well cleared of PP as it

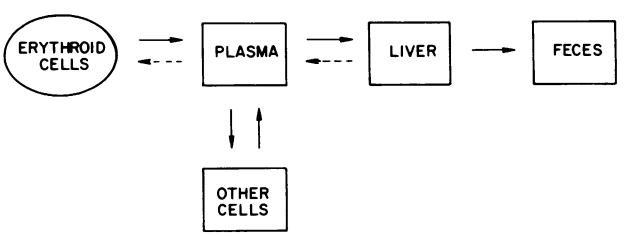

FIGURE 4 Schematic representation of PP transport in human congenital protoporphyria (EPP). The source of the PP, the erythroid cells, is indicated in the oval. The dashed arrows indicate a possible, but unobservably small, flow.

[2] Müller-Eberhard, U., A. Lamola, and M. B. Poh-Fitzpatrick. Unpublished data.

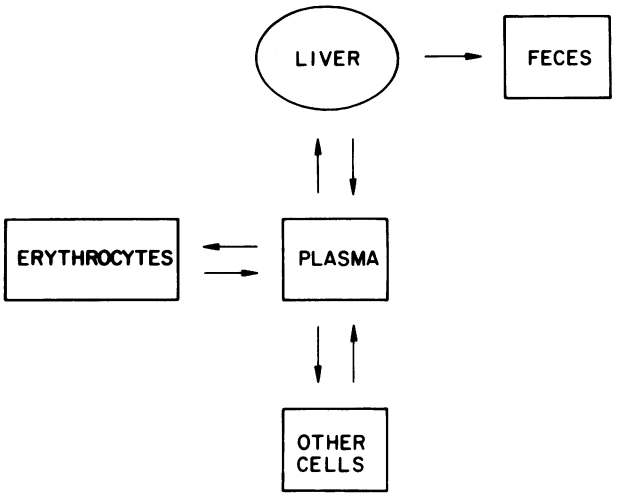

FIGURE 5 Schematic representation of PP transport in mice made chronically protoporphyric with GF. The source of the PP, the liver, is indicated by the oval.

passes through the liver. Only then could the plasma act as an efficient continuous acceptor of erythrocyte PP, working against the preference of PP to bind to cellular hemoglobin. The near equivalence of total daily fecal PP of EPP patients and the amount of PP leaked from the total erythrocyte mass each day, previously pointed out by us and others (7, 8), is justified by the apparent necessity of efficient clearance of plasma-PP by the liver. Liver dysfunction concomitant with hepatic PP deposits could well derive from the large quantities of erythroid-generated PP cleared daily by the liver.

Conclusion. Models for site of production and pathways of diffusion of PP in EPP and GFPP consistent with the present and previously reported data are shown in Figs. 4 and 5. Although some elevation in PP synthesis in the liver of EPP patients cannot be ruled out by the present findings, such liver-formed PP cannot significantly contribute to the circulating PP pool, because the flow of PP is overwhelmingly in the direction from the blood to the feces by way of the liver.

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