Erythropoietic Protoporphyria and Lead Intoxication: the Molecular Basis for Difference in Cutaneous Photosensitivity

II. DIFFERENT BINDING OF ERYTHROCYTE PROTOPORPHYRIN TO HEMOGLOBIN

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ABSTRACT Acidic solvents extract the same porphyrin–protoporphyrin–from the erythrocytes of patients with either erythropoietic protoporphyria or lead intoxication. However, extractable protoporphyrin disappears rapidly, both in vivo and in vitro, from erythrocytes in erythropoietic protoporphyria but slowly, if at all, in lead intoxication. Consistent with these observations, fluorescence spectroscopy revealed that the intracellular state of the erythrocyte protoporphyrin is different in the two diseases. Spectrofluorometric measurements coupled with fractionations and biochemical syntheses showed that in erythropoietic protoporphyria the protoporphyrin is bound as the free base to hemoglobin molecules at sites other than the heme binding sites. In lead intoxication the fluorescent porphyrin is also bound to hemoglobin but is present as zinc protoporphyrin. The data suggest that the zinc protoporphyrin is bound at heme binding sites. Acidic extraction solvents remove the chelated zinc, but zinc protoporphyrin may be extracted intact from erythrocytes with acetone, ethanol, or the detergent Ammonyx-LO.

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INTRODUCTION

Cutaneous photosensitivity due to light absorption by protoporphyrin (PP)[1] is the primary clinical manifestation of erythropoietic protoporphyria (EPP) (1) but not of lead intoxication (PbI) or iron deficiency anemia (FeDA), even in severe cases, though acid-extractable erythrocyte PP levels may be as high in PbI (2) and FeDA (3) as those reached in EPP. The greatly increased levels of PP found in the plasma and feces of patients with EPP are not found for individuals with PbI or FeDA (4, 5). Thus, it has been suggested that PP in the erythrocytes does not provide the basis for the cutaneous symptoms in EPP and that there must be an important extraerythropoietic source in this disease which also accounts for the plasma and feces levels (6).

We have shown (4) that PP diffuses very rapidly from the erythrocytes of EPP patients in vivo but not from the erythrocytes of patients with PbI or FeDA. These findings may obviate a prevalent extraerythropoietic source of PP in EPP in that the photosensitivity may reflect increased turnover of erythropoietic PP with diffusion from the erythrocytes through the plasma into the skin. The lack of photosensitivity in PbI and

[1] *Abbreviations used in this paper:* EPP, erythropoietic protoporphyria; FeDA, iron deficiency anemia; FEP, free erythrocyte porphyrin; Hb, hemoglobin; PbI, lead intoxication; PP, protoporphyrin; ZnPP, zinc PP.

FeDA reflects the absence of PP turnover and of diffusion from the erythrocytes.

The concept that PP leaks from erythrocytes of EPP patients but not from erythrocytes of PbI patients suggests that the intracellular state of PP is different in these two cases. We report here spectroscopic and biochemical evidence that this is indeed the case. Our findings further allow us to propose the following descriptions of the states of the PP in these cells: Almost all of the PP is bound as the free base form to hemoglobin (Hb) molecules at sites other than the sites of heme binding in EPP erythrocytes. In PbI and FeDA, PP is present as zinc PP (ZnPP) (7). The ZnPP takes the place of heme groups in Hb molecules and as such is strongly bound.

METHODS

Blood samples were collected by venipuncture (heparinized tubes) from nine patients with EPP from four different families, from four patients with severe PbI (blood Pb level >90 μg/dl; free erythrocyte porphyrin [FEP] >80 μg/g Hb), six patients with moderate PbI, four patients with severe FeDA (FEP >10 μg/g Hb) as well as from several normal healthy persons (FEP <2.5 μg/g Hb). Erythrocytes were washed in 0.15 M NaCl buffered to pH 7.4 with 0.015 M potassium phosphate ("phosphate-buffered saline," PBS).

Extraction of porphyrins. Porphyrins were extracted from whole blood, washed erythrocytes, plasma, or washed erythrocyte ghosts by a method previously described (8).

Separation of erythrocytes according to age was accomplished by ultracentrifugation on a discontinuous density gradient of Stractan II (St. Regis Paper Co., Tacoma, Wash.) according to a method previously described (9).

Hb solutions were prepared free of stroma by lysing 400 μl washed, packed erythrocytes in 2 ml of 0.01 M *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, pH 7.4, containing 0.001 M EDTA and 0.001 M mercaptoethanol. The stroma was separated by centrifugation for 30 min at 50,000 *g*. The supernatant "stroma-free hemoglobin" was removed and used for electrophoretic studies. For all other purposes Hb solutions were prepared by lysing well-washed erythrocytes in 20 vol of distilled water. The stroma was removed by centrifugation. The supernatant Hb solution was concentrated by vacuum dialysis.

Erythrocyte stroma free of Hb was prepared by washing the pellet of stroma obtained as described above three times in 100 vol of buffer and recentrifuging. The final white pellet contained less than 0.01% of the original erythrocyte Hb.

Preparative Hb electrophoresis was performed on starch granules block, according to Gerald and Diamond (10) by utilizing stroma-free solutions of Hb converted to cyanomethemoglobin.

Gel filtration chromatography of Hb and separated α- and β-chains was performed with a column (2×40 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with 0.01 M phosphate buffer, pH 7. Elution was accomplished with the same buffer. Each fraction was assayed for protein by absorption at 280 nm and for Hb by absorption at 540 nm. The fluorescence spectrum of fractions was measured, as well as the concentration of acid-extractable PP.

Fluorescence spectra and fluorescence excitation spectra were obtained by using a Hitachi Perkin-Elmer MPF2 spectrofluorometer (Perkin-Elmer Corp., Mountain View, Calif.) equipped with a Hamamatsu 446 UR photomultiplier tube (Hamamatsu Corp., Middlesex, N. J.). Wavelength calibration was carried out with the use of a small medium pressure mercury lamp which could be placed in the sample position or at the exciting light entrance slit. The spectra were not corrected for the spectral response of the instrument or the spectral distribution of the xenon source. Maxima of the sharp porphyrin fluorescence bands could be defined within ± 1 nm.

RESULTS

Fluorescence of whole blood and washed erythrocytes. Fluorescence spectra and fluorescence excitation spectra were recorded for samples of blood from patients with EPP, PbI, FeDA, and from normal controls. The whole blood was diluted 500–1,500-fold in PBS and placed in a 1×1-cm fluorescence cuvette. Spectra were also recorded from washed erythrocytes prepared from the same blood samples; the washed, packed erythrocytes were suspended in 1,000–3,000 vol of the buffer. Representative spectra of the diluted whole blood samples are shown in Figs. 1 and 2. The spectra obtained from the washed erythrocytes were identical to the spectra obtained from the whole blood samples from which they were prepared. The relative intensities of the fluorescent emissions from PbI and FeDA blood samples reflected the acid-extractable porphyrin concentrations in the samples.

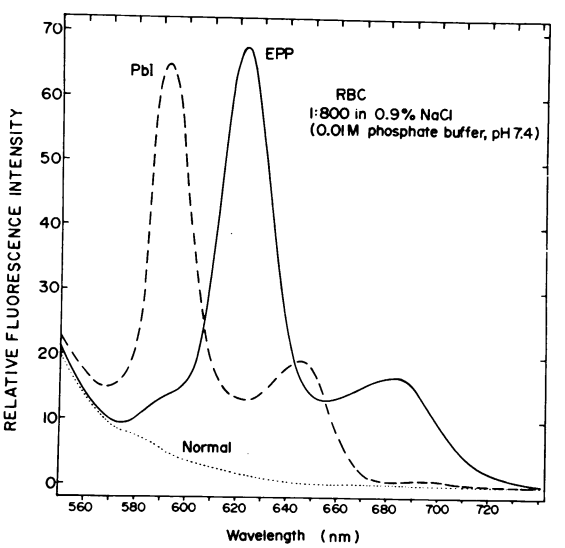

FIGURE 1 The fluorescence spectra of erythrocytes from a normal donor and from patients with EPP and PbI suspended 1:800 in PBS excited at 397 nm and 425 nm, respectively. The spectra are not corrected for the wavelength response of the detector, which was a red-sensitive photomultiplier tube (Hamamatsu 446 UR). Purified Hb fractions from these specimens gave similar spectra.

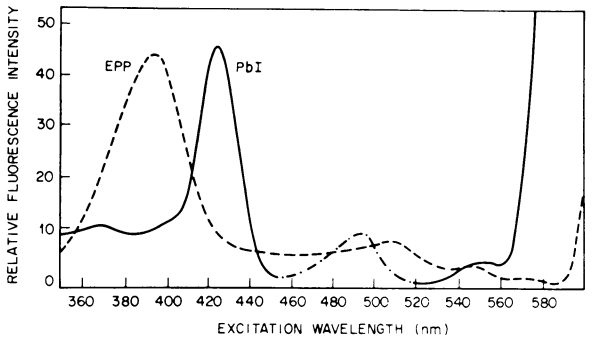

FIGURE 2 Excitation spectra for the fluorescence of erythrocytes from patients with EPP (for fluorescence at 625 nm) and from patients with PbI (for fluorescence at 594 nm). The abrupt rise in the spectra at long wavelengths is due to scattered exciting light. The feature near 490 nm in the PbI spectrum (shown by the dot-dash curve) is unexplained but probably spurious. It was observed in about half of the specimens examined and its shape and maximum were variable. The absorption spectrum of ZnPP has no such band.

The fluorescence spectrum of blood or erythrocytes from PbI patients is typical of a porphyrin, with the major band at 594 nm. The fluorescence excitation spectrum shows a Soret band at 424 nm and a visible band at 550 nm. The spectra obtained from samples of the blood or erythrocytes of FeDA patients were identical to those from PbI patients. Blood or erythrocytes obtained from normal controls either showed no observable porphyrin fluorescence or gave a very weak emission with a maximum near 595 nm. Diluted blood or erythrocyte suspensions obtained from EPP patients exhibited a fluorescence spectrum with the major band at 625 nm. A weak shoulder near 594 nm was usually also observed. The 625-nm emission was found to be associated with an excitation spectrum with a Soret maximum at 397 nm and visible bands at 508, 545, and 574 nm. The weak emission band near 594 nm was found to have an excitation spectrum peak at 425 nm. The spectral data are listed in Table I.

Spectra of erythrocytes of different mean age. Erythrocytes obtained from persons with PbI, FeDA, or EPP were separated by gradient centrifugation into six fractions of different mean age. The fluorescence spectra of the fractions of PbI or FeDA erythrocytes were identical and the intensity (normalized to Hb concentration in the sample) remained constant within experimental error from the youngest to the oldest fraction. In contrast, the intensity of the fluorescence at 625 nm from EPP erythrocytes declined by a factor of 40 going from the youngest to the oldest fraction, in excellent agreement with extractable PP levels. While the 625-nm band exhibited this marked decrease in intensity, the 594-nm band remained virtually constant from the youngest to the oldest fraction (as it did in cells from PbI or FeDA blood) (Fig. 3).

TABLE I

Maxima of Fluorescence and Fluorescence Excitation Spectra

Medium	Emission	Excitation
	nm	
EPP erythrocytes or Hb		
PBS	625 (683)	397
0.2% Ammonyx-LO	634 (695)	405
Ethanol extract	632 (693)	403
Acetone extract	631 (692)	400
EPP plasma	634 (694)	405
PbI erythrocytes or Hb		
PBS	594 (645)	424
0.2% Ammonyx-LO	593 (645)	422
Ethanol extract	589 (636)	415
Acetone extract	588 (637)	418
FeDA erythrocytes or Hb		
PBS	594 (645)	424
0.2% Ammonyx-LO	593 (645)	422
PP		
0.2% Ammonyx-LO	634 (695)	405
Ethanol	632 (693)	403
Acetone	632 (693)	401
PBS + Hb A	625 (684)	397
PBS + globin	625 (684)	403
PBS + plasma	634 (694)	405
ZnPP		
0.2% Ammonyx-LO	593 (644)	422
Ethanol	589 (636)	415
Acetone	588 (636)	417
PBS + plasma	590 (640)	416
PBS + Hb A	593 (644)	427
PBS + globin	594 (645)	423

List of maxima in the fluorescence spectra and fluorescence excitation spectra of erythrocytes, purified Hb, and plasma of patients with EPP, PbI, or FeDA dissolved or suspended in PBS, dissolved in 0.2% Ammonyx-LO, or extracted with ethanol or acetone; of PP and ZnPP in PBS, Ammonyx-LO, ethanol, acetone, and in plasma; and of the complexes of PP and ZnPP with Hb and with globin.

Two bands are observed in the fluorescence spectra with the detector employed (Hamamatsu 446 UR photomultiplier tube); the main band maxima are given first and the maxima of the minor bands are given in parentheses. The fluorescence maxima were not corrected for the spectral response of the detector and the excitation maxima were not corrected for the spectra distribution of the light source. Only the Soret band maxima are given for the excitation spectra.

Thus, the difference in spectral characteristics of the two kinds of fluorescent erythrocyte porphyrins is accompanied by a remarkable difference in their lifetime within the erythrocyte. The porphyrin associated with the 594-nm fluorescence, which is greatly elevated in PbI and FeDA, does not appear to decrease during

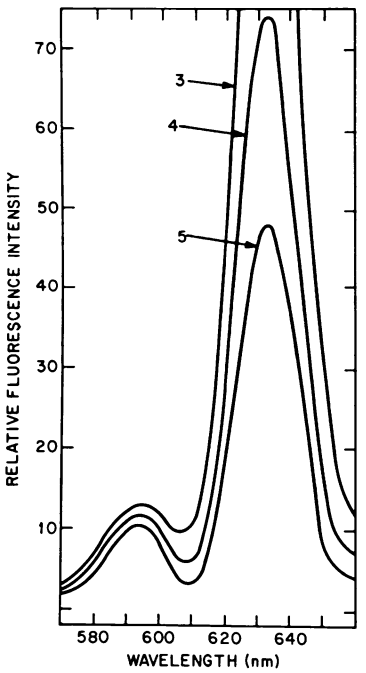

FIGURE 3 Fluorescence spectra of erythrocytes of different mean age obtained from a patient with EPP. The erythrocytes were separated into six fractions of increasing mean age; spectra of three fractions are shown. Samples containing equal amounts of Hb were prepared in 0.2% Ammonyx-LO which shifts the 625-nm EPP-type porphyrin band to 634 nm for better separation from the band at 594 nm. Subtraction of the contribution of the 634-nm band to intensity at 594 nm reveals that the 594-nm band intensity remained virtually constant while the "634"-nm band intensity decreased markedly with erythrocyte age.

the erythrocyte lifetime. In contrast, the porphyrin associated with the 625-nm fluorescence, which is greatly elevated in EPP, decreases rapidly as the erythrocyte ages.

Localization of the fluorescent porphyrins. The acid-extractable fluorescent porphyrin in the bloods of patients with PbI, FeDA, or EPP or of normal controls is associated almost entirely with the erythrocytes. Little or no detectable fluorescence or extractable PP is found in the plasmas of normal persons or of patients with PbI or FeDA. Typically 2-4% of the total extractable PP of EPP blood is found in the plasma. This plasma-bound porphyrin exhibits a fluorescence spectrum with the maximum of the principal band at 634 nm (11). A plasma showing this fluorescence is probably pathognomonic for EPP (11).

Acid extraction of Hb-free stroma prepared from EPP erythrocytes indicated that only 0.25–1.0% of the extractable erythrocyte PP is typically contained therein. Similarly, very little ($\leq$0.5%) of the extractable PP of PbI erythrocytes is found in the stroma. In all three cases, PbI, FeDA, and EPP, the majority ($\geq$98%) of the acid-extractable erythrocyte PP was found to be associated with the stroma-free lysate of the cells which is primarily a solution of Hb.

Gel filtration chromatography of erythrocyte lysate. Stroma-free lysate of EPP erythrocytes was concentrated until the Hb concentration was about 300 mg/ml and loaded onto a column of Sephadex G-100 equilibrated with 0.01 M phosphate buffer, pH 7.0. The column was eluted with the same buffer and the fractions assayed spectrophotometrically at 280 nm (total protein) and at 540 nm (Hb). Fractions were also assayed fluorometrically for porphyrins both by direct measurement of the fractions and by the acid-extraction method (FEP). The elution pattern of fluorescent porphyrin and acid-extractable porphyrin were identical and were exactly superimposable upon the elution pattern of Hb (Fig. 4). The spectrum of each fluorescent fraction was identical.

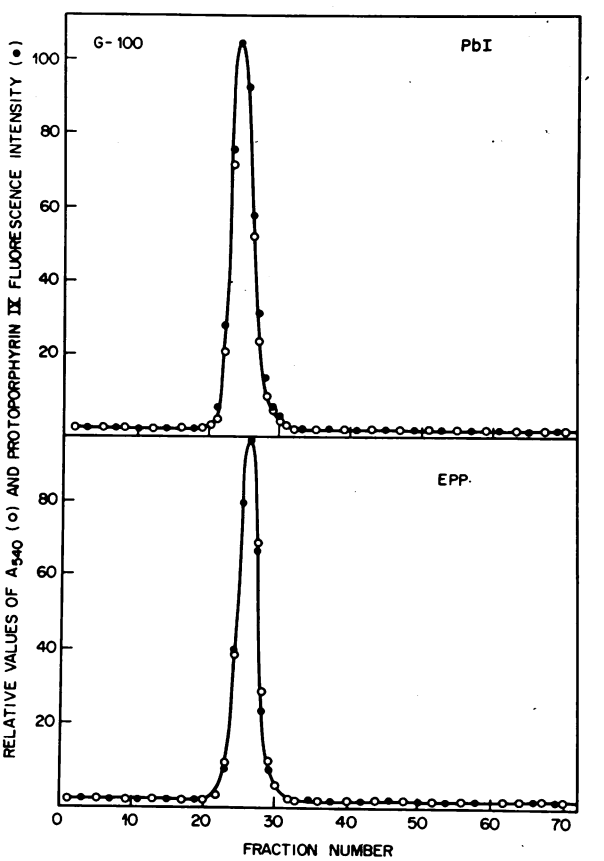

FIGURE 4 Chromatograms of the lysates from erythrocytes obtained from patients with PbI and EPP obtained by using a column packed with Sephadex G-100 eluted with 0.01 M phosphate buffer, pH 7. The relative Hb concentrations (○) and extractable PP concentrations (●) in the fractions are shown.

When this procedure was carried out for lysates of PbI erythrocytes, exactly the same results were obtained; the elution pattern of the PbI-type fluorescent porphyrin was superimposable upon that of Hb (Fig. 4). In both cases the total recovery of acid-extractable PP in the Hb fractions was >90% of that loaded onto the column.

Electrophoresis of erythrocyte lysates. The Hb of stroma-free lysate of EPP erythrocytes was converted to cyanomethemoglobin and then subjected to preparative electrophoresis on starch block. Extractable PP was found associated with the bands of Hb A_1 and A_2. The same observation was made for lysates of PbI and FeDA erythrocytes. When the band of Hb A_1 was divided into three equal zones (fast, intermediate, and slow fractions) the ratio of extractable PP to Hb was nearly constant for all three zones in the case of EPP. In contrast, for PbI and FeDA more than 50% of the extractable PP was found in the fast fraction, and less than 10% was found in the slow fraction. An increased amount of electrophoretically fast-moving Hb (Hb A_a) in patients with PbI has been observed by Charache and Weatherall (12).

Effect of deoxygenation on the fluorescence of the EPP Hb. Two fluorescence cuvettes were filled with a dilute solution ($\sim 2 \times 10^{-6}$ M) of a purified (Sephadex G-100) preparation of Hb obtained from EPP blood. One sample was deoxygenated by slow bubbling of oxygen-free nitrogen until the absorption spectrum showed complete conversion to deoxyhemoglobin; no methemoglobin was apparent. The fluorescence spectrum of the deoxyhemoglobin sample was found to be identical to that from the oxyhemoglobin sample. However, the intensity of the fluorescence from the deoxygenated sample was 0.71 that from the oxygenated sample. When air was admitted into the deoxygenated sample, oxygenation of the Hb occurred rapidly and the fluorescence intensity increased appropriately.

The overlap of the fluorescence spectrum of the EPP-type porphyrin with the absorption spectrum of Hb increases substantially on going from oxy- to deoxyhemoglobin. It was calculated that because of this the rate of electronic energy transfer from excited porphyrin to hemes within the same molecule is expected to increase upon deoxygenation of the hemes, leading to a substantial decrease in the efficiency of porphyrin fluorescence (13, 14).[2] That such an effect is observed indicates that the fluorescent porphyrin is bound to Hb.

A similar calculation based on the fluorescence of the PbI-type predicted an unobservably small effect of deoxygenation of any nearby heme groups. The experiment was not performed for PbI Hb.

Extraction of porphyrins with organic solvents or detergents. Suspensions of erythrocytes (packed cells suspended in an equal volume of PBS) or concentrated Hb solutions prepared from the blood of EPP patients were mixed with 20 vol of acetone and mixed in a Vortex mixer for ½ min and then centrifuged. The supernatant acetone solution exhibited a fluorescence spectrum identical to that of an acetone solution of PP (Table I). Typically 80-90% of the porphyrin extractable by the ethyl acetate-acetic acid-HCl technique was extracted by this acetone method. In contrast, the same acetone treatment of erythrocytes or Hb solutions prepared from bloods of patients with PbI or FeDA typically extracted only 10-15% of the acid-extractable PP. The fluorescence spectrum of these acetone extracts was distinctly different from that of an acetone solution of PP (Table I). When the acetone extract was acidified by adding a small amount of concentrated HCl, the spectrum became identical to that of an acetone solution of PP.

Similar results were observed for extraction of erythrocytes or Hb solutions with absolute ethanol. Extraction with ethanol removes 90% of the PP from EPP erythrocytes or Hb, and the extract exhibited a fluorescence spectrum identical to that of an ethanol solution of PP (Table I). Only 50-80% of the acid-extractable PP of PbI or FeDA erythrocytes or Hb is extracted in one pass with ethanol. The fluorescence spectrum of the extract was distinctly different from that of an ethanol solution of PP (Table I).

When erythrocytes are placed in phosphate buffer containing 0.2% Ammonyx-LO (Onyx Chemical Company, Jersey City, N. J.), a nonionic detergent which is chiefly dimethyldodecylamine oxide, lysis is immediate and the stroma is dissolved. The fluorescence spectrum obtained from buffered solutions of erythrocytes or Hb from patients with PbI or FeDA is only slightly shifted when the detergent was added. However, the fluorescence at 624 nm characteristic of EPP blood was shifted to 634 nm upon addition of detergent.

Gel filtration chromatography (Sephadex G-100) of EPP Hb in 0.2% Ammonyx-LO (0.01 M phosphate buffer, pH 7.0) revealed that the detergent dissociates the PP from the protein since the former eluted well ahead of the Hb band. The fluorescence spectrum of the eluted PP or of EPP erythrocytes dissolved in the detergent was identical to that of a solution of PP in the detergent (Table I). Sephadex chromatography showed that 0.2% Ammonyx-LO also efficiently dissociates PP from the Hb prepared from the bloods of patients with PbI or FeDA. However, the fluorescence

[2] Details of the energy transfer calculation are not given here. The calculation made use of the Förster energy transfer model. A simple treatment of this model was provided by Lamola (15).

spectrum of this purified extract is different from that of a solution of PP in the detergent (Table I).

Separation of α- and β-chains. Samples of Hb prepared from blood from patients with EPP and patients with PbI were reacted with *p*-hydroxymercuribenzoate by the method of Bucci and Fronticelli (16) to produce the α- and β-chains as the *p*-mercuribenzoate derivatives. The α-chains and β-chains were then separated by chromatography on a *O*-(carboxymethyl) cellulose column by using 0.01 M phosphate as eluant with a pH gradient from 6.7 to 8.0. The fluorescence spectrum and amount of acid-extractable porphyrin were determined for each fraction, as well as for the untreated Hb and the unfractionated chains.

The fluorescence spectra of the porphyrin-containing fractions were identical to that of the Hb sample from which they were obtained. The main difference between EPP chains and PbI chains in this experiment was in the recovery of acid-extractable PP. Whereas 80-90% of the Hb-associated PP was recovered in the separated chains in the case of PbI, only 10-30% was recovered in the case of EPP, reflecting a looser binding of the pigment. It appeared that the porphyrin lost from EPP chains remained on the column packing.

Preparation of properties of porphyrin-Hb and porphyrin-globin complexes. An equimolar quantity of PP dissolved in a minimum amount of methanol was added to a 0.1 mM solution (0.01 M phosphate buffer, pH 7.0) of purified Hb A obtained from the blood of a normal donor. The mixture was slowly mixed overnight at 4°C and then passed through a Sephadex G-100 column. The Hb fractions were collected and pooled. These exhibited fluorescence (624-nm maximum) and fluorescence excitation (397-nm Soret) spectra which were indistinguishable from those of EPP Hb.

PP globin was prepared by adding a solution of PP in 0.01 M phosphate buffer, pH 7.0, to a solution of apohemoglobin, prepared by method of Rossi-Fanelli et al. (17) in the same buffer; the final PP concentration was generally on the order of 10^{-4} M and the apohemoglobin was always in excess. The mixture was slowly mixed overnight at 4°C. The fluorescence and fluorescence excitation spectra were found to be independent of the porphyrin/globin ratio from $\frac{1}{10}$ to $\frac{1}{2}$. While the fluorescence spectrum was indistinguishable from that of EPP Hb, i.e. 624-nm maximum, the fluorescence excitation spectrum showed a Soret maximum at 403 nm. The latter corresponded to the Soret maximum observed in the absorption spectra of these preparations. The spectral characteristics agree exactly with those reported in the literature for similar preparations (13, 18-20).

Complexes of zinc PP (ZnPP) with Hb and with apohemoglobin were prepared in similar fashions (13). The ZnPP-Hb complex, ZnPP-globin (13), and PbI-Hb showed indistinguishable fluorescence (595-nm maximum) and fluorescence excitation (Soret maximum near 425 nm) spectra.

DISCUSSION

In the absence of a difference in the rate of transport across the erythrocyte membrane, a large difference in the rate of leak of PP from the erythrocytes of individuals with EPP and those of individuals with PbI or FeDA demands a difference in the state of the acid-extractable PP within the erythrocytes. That, in fact, this is the case is indicated from the great difference in the spectral characteristics of the fluorescence from these cells. A porphyrin fluorescence band at 595 nm is associated with PbI or FeDA while fluorescence at 625 nm is associated with EPP. Erythrocytes from EPP patients and normal healthy donors sometimes exhibit a weak fluorescence at 595 nm. Examination of the fluorescence from EPP red cells as they age revealed that the 595-nm-type fluorescence intensity remains virtually constant while the 625-nm-type fluorescence intensity decreased drastically in accordance with the rapid decrease in extractable PP in a form which does not disappear (leak) from the red cell and that the 625-nm fluorescence band is associated with PP which rapidly disappears (leaks) from the erythrocyte.

The data obtained from several experiments (gel filtration chromatography, electrophoresis, spectral studies) clearly indicate that in both EPP and PbI nearly all of the acid-extractable PP is bound to Hb molecules. The simplest model which explains these observations is that the PP is bound differently to the Hb in the two diseases and that extraction with acidic solvents effectively removes the porphyrin. Extraction of EPP or PbI Hb with acetone, or ethanol, or the detergent Ammonyx-LO also removes the fluorescent porphyrin from the protein. However, in these nonacidic solvents the porphyrin extracted from EPP Hb is clearly different from that extracted from PbI Hb, as evidenced by spectral properties. An easy rationalization of these observations is that one of the porphyrins chelates a metal ion which is easily removed by the acidic extraction solvents. In a separate paper (7) two of us presented data which show that the fluorescent porphyrin associated with PbI is ZnPP while in EPP the fluorescent erythrocyte PP is in its free base form (no metal ion chelated). Extraction with acidic solvents releases the zinc ion and so free PP is obtained.

It should be pointed out that the fact that the abnormally elevated porphyrin in PbI is ZnPP may not in itself be sufficient to explain the absence of photosensitivity of PbI patients since ZnPP can act as photosensitizers much like free base porphyrins (21). At

this point the question becomes, is the only difference between the fluorescent porphyrins of EPP and PbI the chelation of a zinc ion in the latter with the binding site on the Hb the same in both cases, or is there also a difference in the protein binding site?

PP globin was synthesized according to a method well described in the literature. There is a large body of evidence to indicate that in this complex the PP occupies the heme binding site (13, 17–20). Hb, with all heme sites occupied by hemes, also binds PP, necessarily at extraheme sites. The PP globin and PP Hb can be distinguished on the basis of fluorescence excitation spectra; the Soret maximum of the former is at 403 nm, in agreement with the literature value, while the latter occurs at 397 nm. That for EPP Hb is at 397 nm. PP bound to a variety of proteins exhibits a Soret maximum at 397 nm. Thus, we conclude that the fluorescent PP of EPP is bound to Hb molecules at site(s) other than heme sites (see Fig. 5).

We have not been able to distinguish between ZnPP in a heme binding site and bound to an extraheme site by means of fluorescence. However, there is evidence to suggest that the ZnPP is bound at heme sites. First, the ZnPP is not easily extracted by organic solvents which very efficiently extract PP from EPP Hb. Second, unlike the PP of EPP Hb, the ZnPP remains bound to individual Hb chains after they are separated. These observations can be taken to indicate an interchain binding site for the PP of EPP Hb and an intrachain binding site for the ZnPP of PbI Hb.

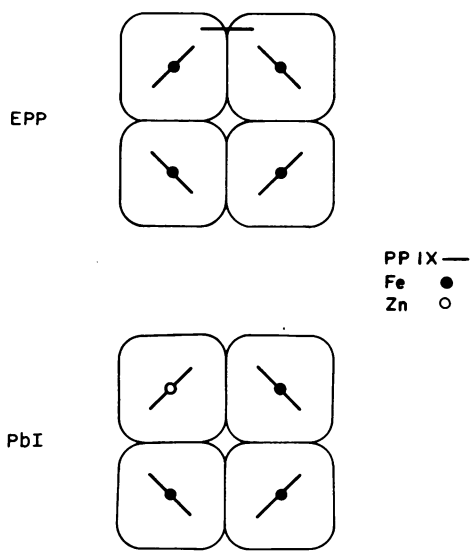

FIGURE 5 Schematic representation of the models for the altered Hb in EPP and PbI. The four subunits of Hb are represented by the squares with rounded corners; PP groups are represented by heavy lines, iron atoms by solid circles, and a zinc atom by an open circle. The fluorescent porphyrin in EPP is in its free base form and occupies an intersubunit site while all the heme sites are normally occupied. The fluorescent porphyrin in PbI is ZnPP which occupies a heme binding site.

Finally, we can offer the following theoretical argument for locating the ZnPP in heme sites: The zinc ion is one of the few metal ions which is readily chelated by PP in an aqueous environment (reference 22, p. 35). At the same time the zinc ion is readily removed from ZnPP (reference 22, pp. 31-34). Thus, it is reasonable to expect a dynamic, albeit slow, equilibrium between free base PP and ZnPP in common pools. The fact that free PP predominates in EPP and ZnPP predominates in PbI could be explained on the basis of a difference in the porphyrin binding sites which tips the equilibrium in one or both cases.

Heme synthesis is decreased in FeDA because of the iron deficiency and in PbI because lead inhibits ferrochelatase activity (23, 24). If PP and globin syntheses proceed in the normal ratio, then porphyrin and globin with unoccupied heme sites are expected to accumulate. In EPP, heme synthesis appears normal despite a large overproduction of PP (1, 25). Heme sites in globins are expected to be occupied as normally by heme. Our data show that the excess PP in EPP erythrocytes binds to Hb molecules (in its free base form) at site(s) other than the heme site. This binding site(s), which appears to prefer free base PP or is unable to significantly disturb a free base $\rightleftharpoons$ zinc porphyrin equilibrium lying on the free base side, is also available in PbI or FeDA red cells. However, in the latter cells the porphyrin is bound to Hb as ZnPP. What is indicated is some other site which prefers ZnPP over the free base form. Such a site would be the heme binding sites, which are expected to be abnormally unoccupied in PbI and FeDA. Zinc is pentadentate and the proximal histidine in the heme pocket of globin represents an ideal fifth ligand to stabilize the ZnPP. Thus, ZnPP is accumulated and stabilized in heme sites in PbI Hb (see Fig. 5). Although our data do not prove this model they are fully consistent with it.

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