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

I. DIFFERENT RATES OF DISAPPEARANCE OF PROTOPORPHYRIN FROM THE ERYTHROCYTES, BOTH IN VIVO AND IN VITRO

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ABSTRACT In lead intoxication photosensitivity is usually absent, despite concentrations of protoporphyrin in the erythrocytes equal to or greater than in erythropoietic protoporphyria. Profound differences in the distribution of protoporphyrin in aging erythrocytes were demonstrated by age-dependent fractionation of cells on discontinuous density gradients. In erythropoietic protoporphyria the concentration of protoporphyrin declined extremely rapidly with erythrocyte age; the bulk of the protoporphyrin was lost in less than 3 days and the concentration of fluorescent erythrocytes in the gradient paralleled the decline of protoporphyrin. In lead intoxication the protoporphyrin concentration declined only slightly with cell aging and erythrocytes of all ages fluoresced. In the bone marrow from a patient with erythropoietic protoporphyria all reticulocytes, but only occasional late normoblasts, fluoresced, suggesting a single population. Sterile incubation in plasma (pH 7.5) demonstrated rapid diffusion of protoporphyrin from the erythrocytes in erythropoietic protoporphyria, but not in lead intoxication. Plasma protoporphyrin was elevated in erythropoietic protoporphyria, but not in lead intoxication. Estimates of the daily loss of protoporphyrin from erythropoietic tissue in erythropoietic protoporphyria suggested an order of magnitude similar to the total blood protoporphyrin. Therefore, it is not necessary to postulate a preponderant extraerythropoietic source to explain the amount of fecal excretion. A significant amount of the diffused protoporphyrin probably reaches the skin with resulting photosensitivity. In contrast, in lead intoxication protoporphyrin remains within the erythrocyte throughout its life span; there is no diffusion into the plasma and hence no photosensitivity.

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INTRODUCTION

An elevated concentration of protoporphyrin (PP)[1] may be extracted with acid organic solvents from the erythrocytes of individuals with the genetic disorder erythropoietic protoporphyria (EPP) (1) and of individuals with chronic lead intoxication (PbI) (2). In EPP the values observed may be up to 100 times greater than normal; in PbI PP concentration in the erythrocytes increases exponentially as the blood Pb level increases and in severe cases it reaches values even greater than in EPP (3). A less pronounced increase in PP concentration is also observed in the erythrocytes of patients with iron deficiency anemia (FeDA) (4, 5).

[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; EPP, erythropoietic protoporphyria; FeDA, iron deficiency anemia; FEP, free erythrocyte porphyrins; Hb, hemoglobin; PbI, lead intoxication; PP, protoporphyrin.

Patients with EPP exhibit severe and often incapacitating cutaneous photosensitivity (1), but such symptoms are usually absent in patients with PbI, although the levels of PP in the erythrocytes are similarly elevated. The discrepancy between the clinical manifestations associated with a superficially similar biochemical finding in the erythrocytes may suggest various interpretations: PP in the erythrocytes bears no causal relationship to cutaneous photosensitivity (6); there is a preponderant extraerythropoietic source of PP in EPP (7); there is a difference in the physical state of PP in the erythrocytes in these conditions; there may be an effect of lead that mitigates the photosensitive response.

The present study was directed toward an evaluation of the hypothesis that photosensitivity reflects increased turnover of PP in EPP, with diffusion from the erythrocytes through the plasma into the skin, and that lack of photosensitivity in PbI reflects absence of PP turnover and absence of diffusion from the erythrocytes.

It is known that the acid-extractable porphyrins ("free erythrocyte porphyrins," FEP) in the erythrocytes in both EPP and PbI consist primarily of protoporphyrin IX (>95%) (6). The concentration of FEP may be precisely measured from minute volumes of erythrocytes (8). Although the concentration of PP in the erythrocyte is similar in EPP and PbI, several other findings suggest a difference in porphyrin metabolism. PP is increased in the feces in EPP (1) but not in PbI (9). Although both EPP and PbI erythrocytes fluoresce under ultraviolet light microscopy, the fluorescence is homogeneous in PbI (10), while in EPP only a percentage of the erythrocytes fluoresce, some with extreme intensity (1). Plasma PP is increased in EPP patients, but no data are available on plasma PP levels in PbI patients.

METHODS

Blood samples were collected in heparin from nine patients with EPP from four different families (clinical details of these patients have been reported) (11); four patients with severe PbI (12) (blood Pb level >90 μg/dl; FEP > 80 μg/g hemoglobin [Hb]) and six patients with moderate PbI (12) (blood Pb 60–80 μg/dl; FEP > 20 μg/g Hb) (none of these patients had concomitant FeDA); six patients with severe iron deficiency (FEP >10 μg/g Hb); and eight normal, non-iron-deficient, hospital employees (FEP < 2.5 μg/g Hb).

Measurement of porphyrins in erythrocytes. FEP were extracted and measured in whole blood or washed erythrocytes according to the technique previously described (8). Since in EPP, PbI, and FeDA, almost all of the increased total porphyrin is PP, FEP concentration is essentially equivalent to the concentration of PP. Even in normal erythrocytes, other porphyrins, mainly coproporphyrin, represent less than 5% of the total (13). Since in most experiments it was not feasible to measure the hematocrit of the sample, the concentration of FEP was expressed in micrograms per gram Hb rather than in micrograms per deciliter erythrocytes. The Hb concentration of whole blood, washed erythrocytes, or erythrocyte lysates was measured according to Van Kampen and Zijlstra (14).

Measurement of porphyrins in plasma. Porphyrins in plasma were extracted by adding to 0.5 ml of plasma 0.3 ml of 5% Celite (Johns-Manville Products Corporation, New York) in saline and 2 ml of ethyl acetate: acetic acid (4:1 vol/vol). The test tube was then agitated for 10 s on a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.), centrifuged in a table centrifuge, and the supernate transferred to another tube. From this point on the procedure was the same as for erythrocyte porphyrins (8). Plasma Hb was measured according to Crosby (15) with *o*-tolidine rather than benzidine used for color development.

Measurement of fluorescent cells. Fluorescent cells were demonstrated in wet preparations of erythrocytes or bone marrow cells suspended in saline buffered to pH 7.4 with 0.015 M potassium phosphate. The most intense red fluorescence was elicited by vertical illumination with a Zeiss Photomicroscope II (Carl Zeiss, Inc., New York) equipped with a 250-W mercury vapor lamp (replacing this light source with a 100-W quartz-iodine lamp (16) resulted in a large reduction in the percentage of fluorescent cells observed). A 2-mm BG 12 filter (Carl Zeiss, Inc.) was used in the excitation beam, and a 540-nm cut-off filter was used as an emission barrier. In the case of EPP, counts of fluorescent cells were obtained both from erythrocytes and bone marrow cells. Because of the rapid fading of fluorescence, visual counts could not be obtained and it was necessary to count cells from photographs taken in standard conditions. For this purpose two photomicrographs of individual fields were obtained, one under phase illumination to count the total number of cells and another under fluorescent epillumination to count the fluorescent cells. Photomicrographs with the phase optics were obtained by utilizing the automatic exposure of the instrument; photomicrographs of fluorescent cells were obtained with a fixed exposure time of 15 s, excluding the ocular prism from the light path. The exposures were recorded on Kodak high-speed type B Ektachrome film, overdeveloped to a rating of 640 ASA. The fluorescent cells were classified from the photographs as "intensely fluorescent" or "fluorescent," the latter being all cells in which any degree of fluorescence was detectable. The percentage of fluorescent cells was calculated from the ratio of the number of fluorescent cells counted to the total number of cells present in the same field. In the case of bone marrow suspensions cell types were identified by supravital stain with new methylene blue. Preparations of bone marrow cells devoid of all erythrocytes were obtained by centrifugation of the original suspension on a layer of Stractan II (St. Regis Paper Co., Tacoma, Wash.) of specific gravity 1.072 (17). In the case of PbI and of FeDA erythrocytes, the fluorescence was too faint and too rapidly evanescent to permit microphotography; only a visual impression could be obtained that the fluorescence was homogeneously distributed in almost all erythrocytes (10).

Separation of erythrocytes according to age. Separation was achieved by ultracentrifugation on a discontinuous density gradient, according to the technique originally described (18), modified to utilize Stractan II instead of bovine crystalline serum albumin (17). Each sample was separated in six or seven fractions. Age-dependent separation in the gradients was verified by measurement of erythrocyte pyruvate kinase and determination of the reticulocyte percentage in the various layers. These were not significantly different for any of the groups studied from the values ob-

served in previous similar experiments (18). The relationship between position in the gradient and mean cell age was computed according to the mathematical model previously described (18), with the aid of a microcomputer (no. 9830, Hewlett-Packard Co., Palo Alto, Calif.).

RESULTS

Concentration of PP in erythrocytes of different mean age. This was measured in groups of cells of progressively increasing mean age by two different methods: the measurement of extractable porphyrins (micrograms FEP per gram Hb), which reflects the average level of PP in groups of cells, and the count of fluorescent cells, which reflects the presence of excessive PP in individual cells.

Concentration of PP measured as extractable porphyrins. The results are shown in Fig. 1. The concentration of PP in EPP erythrocytes declined extremely rapidly with cell age compared to the rate of decline in normal, PbI, and FeDA erythrocytes.

In EPP erythrocytes, the PP concentration declined exponentially with age of the cell. The estimated PP concentration in erythrocytes of age 0 days appeared to be at least 200 times greater than in erythrocytes of age 120 days. At least two exponential rates of loss were apparent: a faster rate for the youngest erythrocytes (uppermost fractions) and a much slower one for the oldest (lowermost fractions). More than one rate of decline was equally apparent when either individual fractionation experiments or pooled data from all the runs were analyzed. In Fig. 1 the data were analyzed in terms of two exponential rates of decline, computed by utilizing from each experiment only the uppermost or the lowermost layers, respectively. However, the data could be interpreted in terms of multiple exponential rates of decline, with $t_{\frac{1}{2}}$ values ranging from a few hours to several days. The bulk of the PP is lost from the erythrocytes within 1–3 days. These findings suggest either multiple PP pools within each erythrocyte with different rates of disappearance or multiple populations of erythrocytes possessing different rates of PP loss.

By contrast the rate of decline of PP in normal erythrocytes appeared much slower and could be defined by a single exponential rate, $t_{\frac{1}{2}} = 60$ days.

In PbI erythrocytes, the concentration of PP declined exponentially with a single rate of decline which was slower than in normal erythrocytes. The slopes of decline were essentially similar in severe and mild cases ($t_{\frac{1}{2}}$ of PP, 115 and 120 days, respectively) and was thus

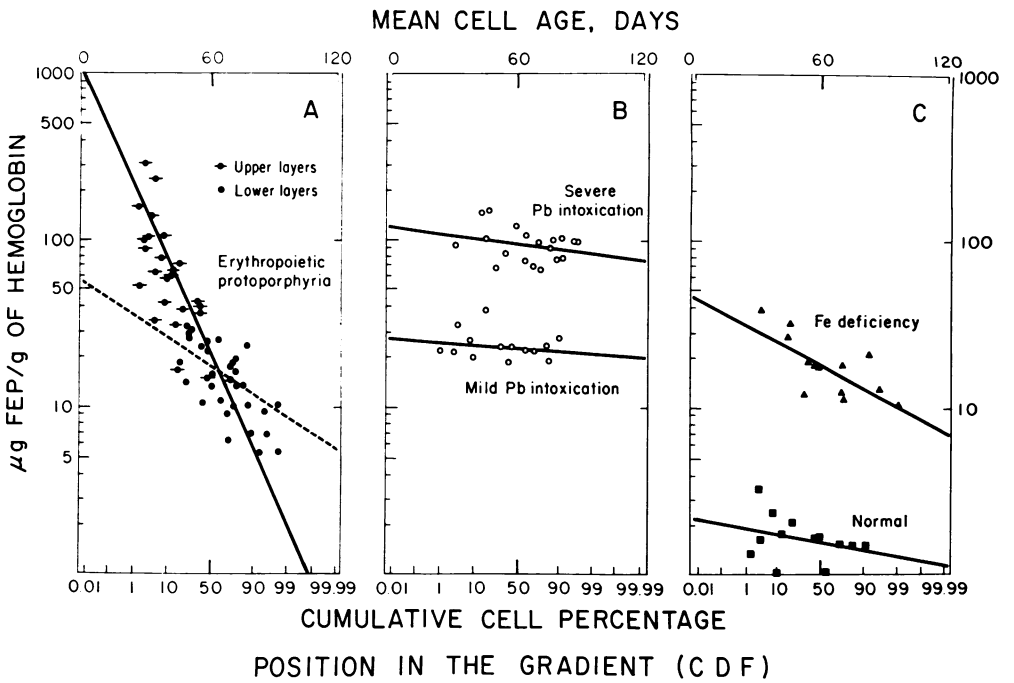

FIGURE 1 Rates of decline of FEP in erythrocyte fractions separated according to age on discontinuous density gradients (17). (A) EPP. Solid line, rate of decline computed from the three uppermost fractions of each gradient; dashed line, rate of decline computed from the three lowermost fractions of each gradient. (B) Upper line, severe PbI (blood Pb > 90 μg/dl); lower line, mild PbI (blood Pb 60–80 μg/dl). (C) Upper line, severe FeDA (Hb < 5 g/dl; transferrin saturation < 5%); lower line, normal individuals. CDF, cumulative distribution function.

independent of the level of PP itself in the two groups. The persistence of PP in PbI erythrocytes was further confirmed by repeating the fractionation according to age of the erythrocytes of a patient with mild PbI after 60 days of intense chelation therapy. In this experiment the elevation of PP persisted in the two oldest fractions, but it had decreased threefold in the two youngest fractions; intermediate groups of cells had intermediate values. These findings confirm that the majority of PP persists in PbI erythrocytes for more than 60 days.

In FeDA erythrocytes, the concentration of PP declined at a rate which was apparently slightly faster ($t_{\frac{1}{2}} = 40$ days) than that of either PbI or normal erythrocytes. This difference does not necessarily reflect an accelerated rate of decline in vivo since the erythrocyte population in FeDA is composed of cells formed at different stages of iron deficiency. Thus, the youngest erythrocytes contain more PP not strictly because of their age, but because they were formed during the most severe period of iron deficiency, usually immediately before diagnosis. Moreover, hypochromic microcytic cells are lighter than normal cells and therefore accumulate higher up in the gradient than do normal cells of the same age (19). These factors contribute toward reducing the estimate of the in vivo $t_{\frac{1}{2}}$ of PP obtained by this method.

Concentration of fluorescent erythrocytes. The results are shown in Fig. 2. In EPP the concentration of fluorescent erythrocytes rapidly declined in the gradient. The rate of decline of intensely fluorescent erythrocytes was slightly slower than the rate of decline of reticulocytes. These findings indicate that the intensely fluorescent cells are very young erythrocytes, which retain intense fluorescence in vivo for a short period after loss of the reticulum. The rate of decline of all fluorescent cells was slower ($t_{\frac{1}{2}} = 20$ days), indicating that residual fluorescence persists in the erythrocytes much longer after the reticulocyte stage. Extrapolation of the slope of decline to 100% reticulocytes indicates clearly that all reticulocytes are intensely fluorescent and that all erythrocytes up to at least 20 days of age retain some fluorescence. These observations exclude the existence of several clones of young erythrocytes with different levels of PP.

By contrast, the fluorescence of erythrocytes from patients with PbI was too faint and evanescent (even when the average PP level was 50 times greater than normal) to permit accurate quantification by photography. It was clear, however, by visual estimation that

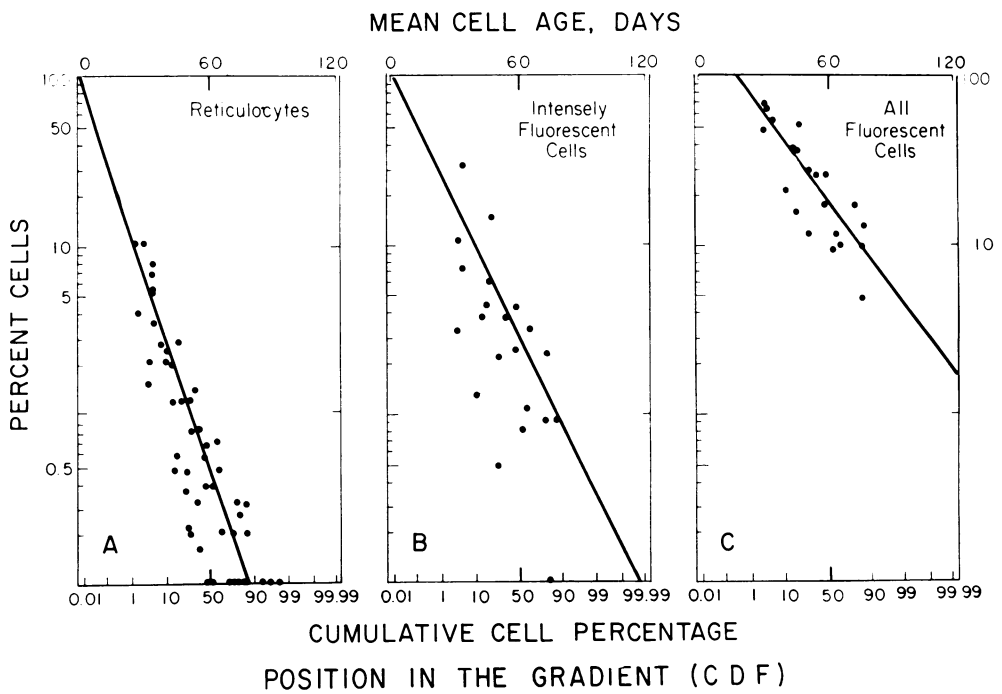

FIGURE 2 Rates of decline of the percentage of reticulocytes and fluorescent cells in erythrocyte fractions separated according to age on discontinuous density gradients (17). (A) Reticulocytes in fractions from EPP patients. (B) Intensely fluorescent cells in fractions from EPP patients. (C) All fluorescent cells in fractions from EPP patients.

the fluorescence was homogeneously distributed in virtually every cell. A double population of erythrocytes, young-nonfluorescent and old-fluorescent, respectively, was observed in the patient with PbI after 60 days of therapy.

The fluorescence also appeared homogeneously distributed in the FeDA erythrocytes, but it was too faint to allow firm conclusion. Normal erythrocytes exhibit no fluorescence detectable under the microscope.

Fluorescence of erythrocyte precursors in the bone marrow of an EPP patient. One EPP patient gave informed consent to have a bone marrow aspiration performed for this study. The bone marrow appeared morphologically normal by Giemsa stain, with a myeloid: erythroid ratio of 2.3 : 1. Examination of a fresh preparation suspended in buffered saline indicated nearly total absence of fluorescence in the normoblasts. Only occasionally could fluorescent cells be detected after removal of all erythrocytes by density gradient centrifugation. Direct count indicated less than 1 in 500 normoblasts which exhibited fluorescence. These were in the most advanced stage of maturation as they all had pycnotic nuclei or only nuclear remnants. By contrast all bone marrow reticulocytes exhibited intense fluorescence.

Concentration of FEP in plasma. The concentration of FEP in plasma ranged between 2.6 and 6.7 μg/dl in the nine EPP patients studied. In the four patients with severe PbI, in the six patients with moderate PbI, and in the six patients with FeDA the concentration of FEP in the plasma ranged between 0 and 0.3 μg/dl. The concentration of FEP in the plasma of the eight healthy volunteers ranged between 0 and 0.3 μg/dl.

The elevated values observed in EPP plasma agree with those reported in the literature (6). The values in PbI and FeDA are in the normal range. These findings demonstrate that, in contrast to EPP, in PbI there is no elevation of plasma FEP, even in the face of extremely elevated levels in the erythrocytes.

Diffusion of PP from erythrocytes in vitro. Packed EPP and PbI erythrocytes from three patients in each group were incubated under sterile conditions with 5 vol of ABO compatible plasma. The plasma was anticoagulated either with acid citrate dextrose (ACD) (final pH of the erythrocyte suspension, 5.7) or with a trace of heparin (final pH of the erythrocyte suspension, 7.5). The cell suspensions were incubated at 37°C in an atmosphere of 95% O_2 and 5% CO_2 in a metabolic incubator, shaken at 20 oscillations per min. Samples were removed at 0 time and at 3, 6, and 18 h. The concentration of PP was measured immediately in the entire suspension, the packed erythrocytes, and the supernatant plasma. Plasma hemoglobin was also measured. The results of a representative experiment are shown in Fig. 3. At pH 7.5, PP diffused from EPP erythrocytes into the plasma at rates between 0.3 and 0.7% of the total per hour. By contrast only approximately 0.1% of the total PP diffused from PbI erythrocytes during the entire period. The amount of PP released into the plasma could not be accounted for by hemolysis, which was minimal under these conditions, even assuming a preferential loss of only those erythrocytes with the highest PP levels. The diffusion of PP from the erythrocyte was found to be pH dependent. In fact only minimal diffusion of PP was observed from EPP erythrocytes suspended in ACD plasma, which has a pH of 5.7. These experiments indicate that PP may diffuse easily from EPP erythrocytes but not from PbI erythrocytes.

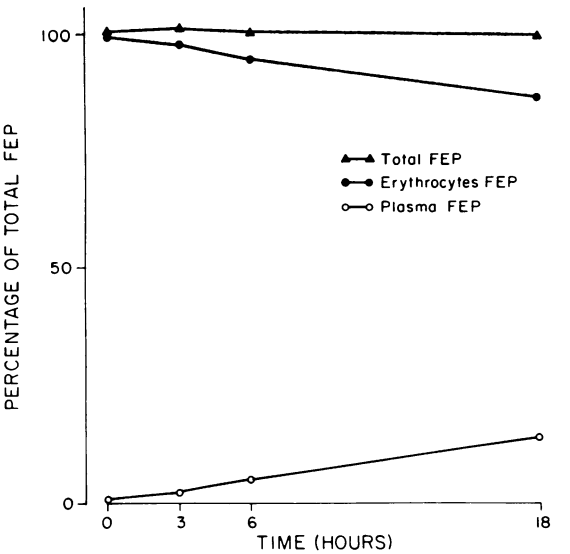

FIGURE 3 Rate of diffusion of FEP from EPP erythrocytes incubated at 37°C under sterile conditions in plasma, pH 7.5. *Total FEP* corresponds to the total amount of FEP in the entire incubation mixture; *erythrocytes FEP* corresponds to the total amount of FEP present in the erythrocytes; *plasma FEP* corresponds to the total amount of FEP present in plasma. Results are expressed as percentage of the total FEP at 0 time. The figure shows a representative experiment; two other experiments yielded essentially similar results.

In similar experiments performed with ACD-anticoagulated plasma (pH 5.7) the percentage of total FEP diffused from the EPP erythrocytes to the plasma at the end of 18 h incubation was $< 2\%$ (not shown). In similar experiments with erythrocytes from patients with severe PbI, the percentage of total FEP diffused from the erythrocytes to the plasma at the end of 18 h incubation was $< 0.1\%$ at either pH 7.5 or pH 5.7 (not shown).

DISCUSSION

A greater concentration of PP in the youngest erythrocytes of patients with EPP had been suggested by the

fractionation experiments of Schothorst et al. (20) and of Clark and Nicholson (21). These findings, however, could not be compared to control observations since the photometric techniques for measurement of porphyrins used in their studies were not sensitive enough for use with small volumes of normal erythrocytes. By contrast, Kaplowitz et al. could not observe any difference in the concentration of fluorescent cells, obtained by visual estimation (22). Langer et al. have recently suggested that in normal erythrocytes the in vivo $t_{\frac{1}{2}}$ of PP is 2-3 wk (23). Their conclusions were based on indirect evidence gathered by measuring the changes in concentration of PP in the erythrocytes that occur during the erythropoietic depression in individuals descending from high altitude. Johnson and Schwartz estimated with the technique of differential hemolysis that the in vivo $t_{\frac{1}{2}}$ of PP in the erythrocytes of a porphyric calf was of the order of 2-3 wk (24). The experiments performed in the present study utilized a technique for separation of cells which can be meaningfully interpreted in terms of in vivo survival (18), in combination with a more sensitive fluorometric method. Our findings clearly indicate that the rate of disappearance of PP from EPP erythrocytes is extremely rapid and of an entirely different order of magnitude than in either PbI, FeDA, or normal erythrocytes. In all the latter types of erythrocytes most of the PP persisted through their life spans. In PbI erythrocytes PP persisted even longer ($t_{\frac{1}{2}} = 120$ days) than in normal erythrocytes ($t_{\frac{1}{2}} = 60$ days). In FeDA the rate of decline in the gradient was slightly greater than in normal erythrocytes. It appears likely that this difference may be due to a selective increase in PP levels in the erythrocyte formed during the height of iron deficiency, immediately before diagnosis, and therefore does not reflect a different rate of in vivo disappearance. Further similarities between PbI and FeDA discussed in the following paper support this interpretation (25).

Our findings indicate that in PbI the content of PP is very nearly the same in erythrocytes of all ages, but in EPP it may differ between young and old erythrocytes by as much as 200 times. In EPP, the different levels cannot be due to the production of different clones of erythrocytes with varying PP content since all reticulocytes have greatly increased levels. Examination of erythrocyte precursors in the bone marrow indicates that accumulation of intense fluorescence begins just before the nucleus of the normoblast is extruded and continues until the mitochondria are lost from the cell. A similar distribution of fluorescence had been reported by Clark and Nicholson in bone marrow preparations from their patients (21).

The PP accumulated in the newly formed erythrocytes rapidly disappears as the cells enter the circulation. These observations cannot be explained by a rapid hemolysis of PP-containing reticulocytes since, except for unique cases (26), EPP patients show no evidence of hemolysis (6). In fact, a rate of hemolysis commensurate with the rapid disappearance of fluorescent cells could not occur without striking reticulocytosis and/or anemia. (All of the EPP patients in this study had normal hematological values and reticulocyte counts.) It appears, therefore, that in EPP an extremely high concentration of PP accumulates in the erythrocytes in the later stages of maturation in the bone marrow and disappears rapidly from circulating erythrocytes.

The very short persistence in vivo of PP in the EPP erythrocyte may be explained either by diffusion from the cell or by intracellular catabolism. Diffusion of PP from EPP erythrocytes into plasma was clearly demonstrated in vitro by sterile incubation of EPP erythrocytes in plasma under controlled conditions, when the pH was 7.5 or above. These findings are at variance with the early observations of Redeker et al. (27), who failed to demonstrate loss of PP from EPP erythrocytes to the plasma. Technical details of the incubation experiments of these authors were not reported. Their inability to observe PP loss from erythrocytes to plasma may have been due to poor sensitivity of the spectrophotometric technique used in their study or to failure to control the plasma pH.

Diffusion of PP from EPP erythrocytes into the plasma had been suspected by the elevation of plasma PP observed in this condition (1). Recently, Sholnick et al. suggested that the majority of plasma PP in EPP may be of hepatic origin, on the basis of in vivo studies using radioactive precursors (7). Their interpretation was based in part on the assumption that diffusion of PP from the erythrocytes into the plasma does not occur, as reported by Redeker et al. (27) and also on the assumption of intraerythrocytic persistence of PP. Schwartz et al. (28) interpreted analogous in vivo isotopic studies to suggest that the increased plasma PP reflects an extremely rapid release of PP from maturing erythrocytes. These authors also demonstrated that, upon incubation in vitro of EPP blood with radioactive precursors, a large proportion of plasma PP becomes radioactive. Since hepatic synthesis can certainly not be the mechanism for labelling of PP in vitro, these experiments indicate rapid diffusion of PP from the erythrocytes. Our data indicate that PP disappears rapidly from EPP erythrocytes in vivo and that it may diffuse in vitro. Therefore it appears reasonable to conclude that in vivo a rapid diffusion of PP occurs in circulating erythrocytes and this may happen at an even faster rate during the last stages of erythrocyte maturation in the bone marrow.

Increased fecal excretion of PP is a feature of EPP,

but does not occur in PbI (9). The daily excretion of PP in EPP may reach the same magnitude as the total PP of the circulating erythrocyte mass (7). This finding has been interpreted as additional evidence that in EPP a preponderant amount of fecal PP may be of extraerythropoietic (hepatic) origin. Our data cannot provide an absolute quantitative estimate of the total daily turnover of PP of erythropoietic nature since the amount of PP that diffuses in the bone marrow before the erythrocytes enter the circulation could not be quantified. In fact, since the rate of PP loss from circulating erythrocytes progressively declined with cell aging, it is likely that diffusion is the greatest from the bone marrow reticulocytes. A reasonable estimate of the daily loss of PP from circulating erythrocytes may, however, be obtained from our data by using the mathematical model described in the Appendix. This shows that for an average PP turnover time of 2 days in EPP erythrocytes, the concentration of PP in day 0 cells should be 60 times greater than the average concentration of PP in the entire cell population. This estimate agrees with the 52:1 ratio obtained from the mean concentration of PP at day 0 estimated by the slope of decline (1,035 μg/g Hb) and the mean average concentration (20.5 μg/g Hb) in the EPP patients studied. The computations in the Appendix also demonstrate that a turnover time of 2 days for PP in circulating erythrocytes results in a daily loss of 4/10 of the total PP content of the circulating erythrocyte mass. This daily loss, combined with the additional loss from bone marrow reticulocytes, could account for most of the daily fecal PP excretion without need to invoke a preponderant hepatic PP formation.

Accumulation of "free PP" in the erythrocytes in PbI and FeDA results from failure to complete the last step in heme synthesis, incorporation of Fe into PP. The mechanism for this metabolic defect is different in these two conditions: in PbI the enzyme involved in this step, ferrochelatase, is directly inhibited by Pb (29); in FeDA the enzymatic machinery is intact, but heme synthesis cannot be completed for lack of iron, the necessary substrate (5). Both conditions are associated with decreased overall PP synthesis (30); the excess of PP is only relative and reflects lack of utilization. On the other hand, in EPP, heme synthesis is not defective (6), and there is a true excess of PP. This excess results from increased synthesis as demonstrated by Porter by in vitro incubation of EPP normoblasts with PP precursors (31).

It must be considered that free PP in the erythrocyte is only a minute fraction of the total amount of PP present as heme. In normal erythrocytes only one molecule of free PP is present for each 30,000 molecules of heme PP. In PbI this ratio increases, in the most severe cases, to one free PP molecule for 300 heme PP molecules (and in FeDA to 1:3,000). Thus, although the concentration of extractable PP in circulating erythrocytes appears greatly increased in PbI (and it is easily demonstrable for diagnostic purposes), the actual amount of free PP is still a small fraction of a reduced overall production. Lichtman and Feldman have demonstrated the absolute depression of heme PP synthesis in PbI by direct measurements of synthetic rates in vitro (30).

Our data demonstrate that in circulating EPP reticulocytes there is on the average one molecule of free PP for each 33 molecules of heme PP. In EPP bone marrow reticulocytes this ratio may increase even further to one molecule of free PP to each 15-20 molecules of heme PP. Therefore, the enormous excess of free PP observed in this syndrome in the peripheral blood may be produced by an increase in overall synthesis of PP of only 5-8%.

The daily fecal excretion of PP varies among EPP patients and may also be influenced by dietary factors (6). The rate of in vivo diffusion of PP from EPP erythrocytes appears to be of the same order of magnitude in all patients. Therefore, the average PP concentration in the erythrocytes and probably also the average daily fecal excretion of PP are in individual EPP patients primarily a function of the individual rate of excessive synthesis of erythrocytic PP. Variations in carbohydrate uptake may influence PP excretion through an effect on erythropoietic as well as hepatic synthesis or alternatively by modification of the rate of disappearance from the erythrocytes.

The studies reported in this paper clarify the mechanism underlying the observed clinical differences of PbI and EPP with regard to cutaneous photosensitivity. In EPP an excess of PP is formed within the erythrocyte in late maturation and rapidly diffuses through the plasma. From this source a significant amount of PP reaches the skin where it induces photosensitivity (6). The low steady-state plasma levels indicate that the PP diffused from the erythrocytes is rapidly cleared from the plasma. The bulk of the PP is excreted via the bile into the feces (6). Hepatobiliary changes usually apparent in the third decade of life are a well-known feature of EPP patients (32); it is likely that these are secondary to the accumulation of the excreted PP. In contrast, in PbI (as in FeDA) there is little turnover of PP, which remains inside the erythrocyte through most of its life span. Hence, PP does not diffuse from the erythrocyte compartment nor is it excreted in the feces. Thus, in PbI, plasma PP levels are normal, skin photosensitivity is absent, and fecal excretion of PP is normal. The experiments described in the following paper (25) indicate that a different

binding of PP to the erythrocytes is the molecular basis responsible for the clinical differences.

APPENDIX

Simplest model for calculating the daily fecal PP excretion in EPP patients from erythropoietic source. Assume PP leaks from circulating reticulocytes and erythrocytes with the same single rate. The concentration of PP in the erythrocyte at time t is related to the concentration at the time the cell enters the circulating blood, PP_o, by the equation:

$$PP(t) = PP_o e^{-t/\tau}, \tag{1}$$

where τ (turnover time) is the time (in days) for PP to drop to $(1/e)PP_o$, and t is the age of the circulating cell (in days). Then, if the age distribution of the erythrocytes is uniform (there is no hemolytic anemia in EPP) and their finite lifespan is 120 days, the average protoporphyrin concentration, $\overline{PP}$, is given by:

$$\overline{PP} = \frac{PP_o \int_0^{120} e^{-t/\tau} dt}{\int_0^{120} dt}, \tag{2}$$

which upon integration gives for $\tau << 120$ days

$$\overline{PP} = \frac{PP_o \tau}{120} \tag{3}$$

For $\tau = 2$ days, $\overline{PP} = (1/60)PP_o$; for $\tau = 1$ day, $\overline{PP} = (1/120)PP_o$.

The amount of protoporphyrin diffusing, PP_l, from each cell in one day is given by:

$$PP_l(t) = P_o[e^{-t/\tau} - e^{-(t+1)/\tau}], \tag{4}$$

so that the average amount diffusing from a circulating cell, $\overline{PP}_l$, is given by:

$$\overline{PP}_l = \frac{PP_o \int_0^{120} [e^{-t/\tau} - e^{-(t+1)/\tau}] dt}{\int_0^{120} dt} \tag{5}$$

$$= \frac{PP_o \tau}{120}(1 - e^{-1/\tau}), \tag{6}$$

which from Eq. 3 becomes

$$\overline{PP}_l = \overline{PP}(1 - e^{-1/\tau}). \tag{7}$$

So that for $\tau = 2$ days, $\overline{PP}_l = 0.39\overline{PP}$; for $\tau = 1$ day, $\overline{PP}_l = 0.63\overline{PP}$.

Our data suggest that the extractable PP disappears from the erythrocytes in vivo with a τ value of 2 days or less. Multiplying by the blood volume this simplest model would predict an average daily fecal PP level equal to at least 0.4 times the total blood PP, assuming, of course, efficient transport to the feces. To this minimum estimate should be added also an even larger amount of PP which could very well diffuse from erythroid precursors in the bone marrow before they enter the circulation. It therefore is quite reasonable to expect a daily excretion in the feces of an amount of PP of the same order of magnitude as is in the total circulating erythrocyte mass.

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