Erythropoietic Protoporphyria: Evidence for Multiple Sites of Excess Protoporphyrin Formation

PERRY SCHOLNICK, HARVEY S. MARVER, and RUDI SCHMID

From the Departments of Dermatology and Medicine, the University of California at San Francisco, San Francisco, California 94122

ABSTRACT A patient with erythropoietic protoporphyria was studied to determine the sites of excess protoporphyrin formation. The patient's protoporphyrin was pulse labeled by the simultaneous administration of the precursors 2-glycine-^{14}C and 3,5-δ-aminolevulinic acid-^{3}H; δ-aminolevulinic acid preferentially labels the hepatic pool. Blood and feces were studied at intervals for the next 14 days. Protoporphyrin was extracted from erythrocytes, plasma, and feces, identified by thin-layer chromatography, and quantitated spectrophotometrically, and its specific activity was determined by liquid scintillation spectrometry. Analysis of the kinetic and isotopic data indicated at least two sources of protoporphyrin, one localized in the erythroid cells, a second in the liver. The liver was responsible for the majority of the excess protoporphyrin. This report thus provides evidence of a genetic porphyria exhibiting an abnormality of porphyrin biosynthesis in at least two tissues. We propose that the disease, erythropoietic protoporphyria, be renamed erythrohepatic protoporphyria.

INTRODUCTION

Erythropoietic protoporphyria (EPP) is a dominantly inherited disorder of heme biosynthesis (Fig. 1) that was first recognized as a distinct entity by Magnus, Jarrett, Prankerd, and Rimington in 1961 (1). Clinically the disease is characterized by a variety of photocutaneous responses induced by light energy of 4000 A, the wavelength at which protoporphyrin absorbs maximally. The diagnosis rests on the demonstration of increased protoporphyrin concentrations in erythrocytes, plasma, and stool. The urinary excretion of porphyrins and porphyrin precursors is normal. In the typical case protoporphyrin-laden erythrocytes in the blood exhibit intense red fluorescence when exposed to near-ultraviolet light. This characteristic has become accepted as the hallmark of EPP (2) and led to the assumption that defective heme biosynthesis in the maturing red cells is the source of the excessive protoporphyrin.

It is apparent, however, that several more recent observations are difficult to reconcile with the concept of a genetic abnormality that is expressed metabolically solely in the erythroid apparatus. First, in a number of instances, marked discrepancies were noted between erythrocyte concentration and fecal excretion of protoporphyrin (3, 4); the quantity excreted daily in the feces exceeded by as much as 50 times the total amount of protoporphyrin in the circulating red cell mass. Since most of these patients had a normal erythrocyte life span, it is apparent that only a small portion of their fecal protoporphyrin could have been derived from senescent red cells. Significant "leakage" of protoporphyrin from intact circulating erythrocytes into the plasma has been ruled out by direct experimental observation (5). Furthermore, no increase in fecal porphyrin excretion is demonstrable in lead intoxication and in iron deficiency (6), both of which are associated with high levels of free red cell protoporphyrin (6–8). Second, investigation of kinships with the disease revealed several affected individuals with elevated fecal porphyrin excretion who failed to exhibit a concomitant increase in red cell protoporphyrin (9, 10). Third, in a patient with EPP studied with glycine-^{15}N, Gray, Kulczycka, Nicholson, Magnus, and Rimington (11) noted that the specific isotope concentration of erythrocyte protoporphyrin could not readily account for the isotope value of the porphyrin in the feces.

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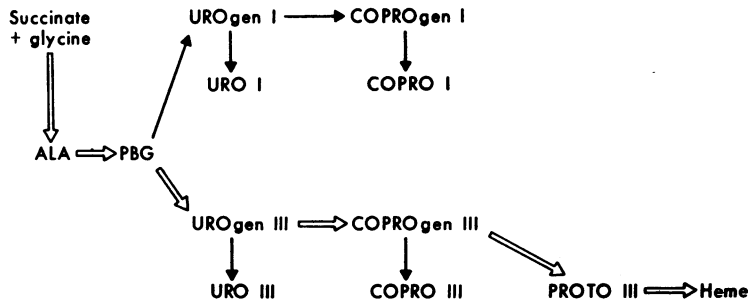

FIGURE 1 Biosynthesis of heme-ALA, δ-aminolevulinic acid; PBG, porphobilinogen; UROgen, uroporphyrinogen; COPROgen, coproporphyrinogen; URO, uroporphyrin; COPRO coproporphyrin; PROTO, protoporphyrin.

In the present study, a kinetic approach was used to identify the sources of the excess protoporphyrin. Such an approach is difficult in normal individuals, but it is feasible in patients with EPP exhibiting marked elevations of protoporphyrin in red cells, plasma, and stool. These increases may be accentuated by starvation (12).

EXPERIMENTAL DESIGN

The experimental protocol was based on the observation that the incorporation of the two isotopic precursors, glycine and δ-aminolevulinic acid (ALA), into porphyrins and heme (Fig. 1) is strikingly dissimilar in the liver and in erythroid cells. This difference exists despite the fact that the biosynthetic pathway for heme is identical in both tissues. Thus ALA would be expected to be a more effective precursor than glycine, because it is utilized almost solely for the biosynthesis of porphyrins and heme (13). Previous investigators have shown that a tracer dose of ALA-^{14}C labels liver porphyrins and heme over 1000 times more intensely then erythrocyte heme, whereas with glycine-^{14}C, labeling of these pigment fractions is more uniform (14, 15). This discrepancy probably results from the relative impermeability of the erythrocyte membrane to ALA (14). In the present study this difference in the fractional incorporation of the two porphyrin precursors was used to delineate the sources of the increased protoporphyrin in EPP. Tracer doses of glycine-^{14}C and of ALA-^{3}H were given simultaneously, and their relative rates of incorporation into the protoporphyrin of erythrocytes, plasma, and feces were determined.

CLINICAL DESCRIPTION

The patient E.W. was a 35 yr old Caucasian man with EPP, weighing 55.5 kg, whose clinical features have been reported previously (12). Physical examination on admission was unremarkable, and no skin lesions were present. Routine laboratory studies were within normal limits. Specific tests yielded the following results: hematocrit, 38%; reticulocyte counts, 0.5–1.0%; plasma iron concentration, 125 μg, and total plasma iron binding capacity, 236 μg/100 ml; BSP retention in 45 min, 6%; SGOT (serum glutamic oxaloacetic transaminase), 36 Karmen units; total serum bilirubin concentration, 1.1 mg/100 ml. The RBC mass was 1.74 liters, and plasma volume was 2.87 liters (albumin-^{131}I). Urinary excretion of ALA and porphobilinogen was normal (16). Erythrocyte, plasma, fecal, and urinary porphyrin concentrations were within normal limits except for the markedly elevated protoporphyrin values noted in Table I.

Before hospitalization the patient's average caloric intake was estimated to have been 2500 calories. On admission he was started on a diet of 800–1120 cal, consisting of 35 g of protein, 35 g of carbohydrate and fat. This relatively fixed, low calorie diet was maintained throughout the study in order to stimulate protoporphyrin production (12). On the 7th hospital day, 0.5 mCi of 3,5-ALA-^{3}H and 0.05 mCi of 2-glycine-^{14}C were administered simultaneously by rapid intravenous infusion. Carmine red was given orally as a stool marker. Blood and feces were collected at frequent intervals over the subsequent 2 wk.

TABLE I
Effect of Diet on Protoporphyrin Values

Day after admission	Caloric intake	Protoprophyrin		
		RBC	Plasma	Stool
		μmoles/100 ml		*μmoles/24 hr*
1	2500 (approx)	3.8	0.09	26.6
4	800	4.5	0.23	51.8
8	1120	4.6	0.33	52.0
12	1120	4.4	0.17	85.0
Normal values*	ad lib.	0.04–0.13	trace—0.04	trace—0.21

* Range of normal values as determined in this laboratory.

TABLE II

Specific Activity of Protoporphyrin (dpm/μmole)

Erythrocyte				Plasma				Feces			
Time*	^{3}H	^{14}C	^{3}H:^{14}C	Time	^{3}H	^{14}C	^{3}H:^{14}C	Time	^{3}H	^{14}C	^{3}H:^{14}C
4.5 hr	897	32	28	30 min	565,800	18,400	30	1 day	27,747	465	59
72 hr	827	214	3.9	60 min	75,400	9900	7.5	2 day	87,363	1322	66
7–14 day	—	—	1.9‡	7 hr	56,400	9100	6.2	3 day	40,370	1004	40
				2 day	14,600	2800	5.1	4 day	16,092	560	28
				4 day	2100	1200	1.7	6 day	7853	320	24
								9 day	4627	210	22
								11 day	896	131	6.8
								13 day	704	109	6.4

* Time refers to interval between intravenous administration of 2-glycine-^{14}C or 3,5-ALA-^{3}H and sample collection.

‡ Ratio determined on a pooled sample collected from days 7–14.

METHODS AND MATERIALS

Porphyrins were extracted from erythrocytes, plasma, urine, and feces by phase partition (17, 18) and quantitated spectrophotometrically or spectrophotofluorometrically using appropriate correction factors (19). After esterification (20) the protoporphyrin isolated by phase partition migrated as a single spot on thin-layer chromatography (21). Free porphyrins and porphyrin esters obtained from the Sigma Chemical Co., St. Louis, Mo., were used as reference compounds. Hemin was prepared from erythrocytes (22) and recrystallized (23).

The specific radioactivity of the isolated porphyrins and hemin was determined by liquid scintillation spectrometry in the following systems: (*a*) dispersion in thixotropic gel (24), (*b*) solution in Hyamine-methanol, and (*c*) combustion (25) and subsequent counting of the trapped radioactivity in Hyamine-methanol. Background counts were determined with unlabeled porphyrins prepared in the same manner. The counting efficiency for each sample was determined with toluene-^{14}C or -^{3}H as internal standards; the efficiency averaged 60% for carbon-14 and 16% for tritium. 3,5-ALA-^{3}H (400 mCi/mmole) and 2-glycine-^{14}C (27.5 mCi/mmole) were purchased from the New England Nuclear Corp., Boston, Mass.

RESULTS

The data obtained in analyzing the radioactivity in the protoporphyrin of the circulating erythrocytes, the plasma, and the feces will be considered in the following three ways: (*a*) the absolute incorporation of the isotopic tracers into each porphyrin fraction (specific activity), (*b*) the changes in specific activity in samples collected serially during the period of observation ("rate of isotope incorporation"), and (*c*) the ratio of the specific ^{3}H activity to the specific ^{14}C activity of each sample (specific activity ratio).

The protoporphyrin of the erythrocytes exhibited a relatively low specific activity (Table II). The rate of isotope incorporation was slow; with 2-glycine-^{14}C the specific activity at 72 hr was higher than that at 4.5 hr. The specific activity ratio which initially was 28, fell to 3.9 in 72 hr and was 1.9 in the pooled sample that represented erythrocytes collected between 7 and 14 days. Red cell hemin isolated from this same sample had a specific activity ratio of 1.9.

In the plasma the protoporphyrin displayed an entirely different labeling pattern (Table II). The specific activity was very much higher than in the red cells. The rate of isotope incorporation was very rapid, but the radioactivity also disappeared quickly from this protoporphyrin fraction. For example, after 72 hr, when the specific ^{14}C activity of the erythrocyte protoporphyrin was still rising, the corresponding value of the plasma protoporphyrin had fallen to less than 20% of the initial value, and the specific ^{3}H activity was less than 2% of the recorded peak value (Table II). Coincident with this was a rapid change in the specific activity ratio which in 4 days decreased from an initial value of 30 to 1.7.

The labeling pattern of the fecal protoporphyrin differed strikingly from that of both the plasma and the red cells. The specific activity was lower than that in the initial plasma sample but very much higher than that in the erythrocytes. The rate of isotope incorporation was rapid with significant label appearing on the 1st day even before the excretion of the carmine red marker. On the other hand, the disappearance of radioactivity from the fecal protoporphyrin was distinctly slower than in the corresponding plasma samples. The specific activity ratio in the initial samples was the highest recorded, and it declined more slowly than that in either the plasma or red cells.

DISCUSSION

In interpreting these results, it is assumed that the patient had a single erythrocyte population. It should be noted that protoporphyrin is excreted solely by way of the bile and feces; urine contains no protoporphyrin

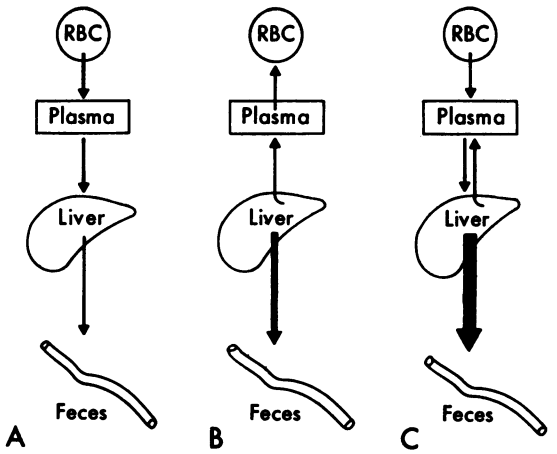

FIGURE 2 Alternative models describing the source(s) of excess protoporphyrin in erythropoietic protoporphyria.

(26). Hence fecal protoporphyrin represents the sum of all protoporphyrin formed at all sites within the body.

As discussed earlier, several observations suggest that the excessive protoporphyrin produced in this disease does not originate solely from the erythroid cells, but probably is derived also from other sources. Among these the liver is a prime suspect because (*a*) it is a site of very active porphyrin and heme biosynthesis (27, 28), (*b*) it is the principal site of porphyrin overproduction in several other types of porphyria (29, 30), and (*c*) in EPP there is a reciprocal relationship between caloric intake and porphyrin excretion, an effect which may be explained by carbohydrate mediated suppression of hepatic porphyrin production (12). The present results will be analyzed in terms of two extreme models, the first of which assumes that all excess protoporphyrin is derived from the erythroid cells, and the second, that the liver is the sole source of the protoporphyrin. A third model is proposed which embraces both extremes.

If all excess protoporphyrin were derived from the erythroid cells, as postulated in model *A* (Fig. 2), the amount and the specific activity of the excreted protoporphyrin should bear a direct relationship to the respective values in the red cells. The following findings are difficult to reconcile with this model. (*a*) The daily fecal protoporphyrin excretion (approximately 50 μmoles) was comparable in magnitude to the protoporphyrin content of the total circulating red cell mass (approximately 46 μmoles). In order to account for this massive porphyrin excretion, all erythrocyte protoporphyrin would have had to turn over at least daily, an assumption which is unlikely in the absence of demonstrable hemolysis. (*b*) The plasma protoporphyrin exhibited a labeling pattern strikingly different from that of the erythrocyte protoporphyrin, a finding that is inconsistent with the concept that the former originates from the latter. (*c*) With both isotopes, the specific activity of the fecal protoporphyrin was very much higher than the corresponding values in the erythrocyte protoporphyrin (Table II). (*d*) The specific activity ratio of the fecal protoporphyrin was higher than that in the erythrocytes, which is in direct conflict with the concept that the red cells are the sole source of the excess protoporphyrin.

Model *B* postulates that all excess protoporphyrin is derived from the liver with a fractional reflux into the plasma and to the red cells (Fig. 2). This assumption is inconsistent with the following findings in the red cells: (*a*) the specific activity ratio of erythrocyte protoporphyrin was markedly different from that of the plasma and feces (Table II), and (*b*) the rate of isotope incorporation into erythrocyte protoporphyrin was much slower than into fecal and plasma protoporphyrin. In fact, the specific ^{14}C-activity in the red cells was still increasing at 72 hr, at a time when the corresponding values in plasma and feces were declining. Thus, model *B* appears untenable because of its failure to account for the labeling pattern of the erythrocyte protoporphyrin.

Since the requirements of neither of these two models are satisfied by the present findings, it is apparent that the excess protoporphyrin must be derived from more than one source (model *C*, Fig. 2). The erythrocytes undoubtedly are one of these sources, as this protoporphyrin fraction exhibited an over-all labeling pattern that can only be explained by porphyrinogenesis *in situ*. This is particularly evident from the low incorporation of ALA-^{3}H into red cell protoporphyrin as compared with the fecal and plasma porphyrin fractions. The initial, rapid appearance of ^{3}H activity in the erythrocyte protoporphyrin fraction probably reflected absorption of small amounts of plasma protoporphyrin on the red cell membrane (31).

Direct localization of the second source of excess protoporphyrin formation is more difficult, but the present findings and the considerations cited earlier strongly implicate the liver. Most compelling among these is the finding that the specific activity ratio of the fecal protoporphyrin was not mirrored by that in the plasma (Table II). This suggests that part of the fecal protoporphyrin was formed in the liver and excreted directly into the bile without gaining access to the plasma. Quantitative considerations based on model *C* (Fig. 2) and taking into account the observed differences in specific activity, the pool size in red cells and plasma and the amount of protoporphyrin in the stool imply that at least 80%, and probably more, of the fecal protoporphyrin must have been of hepatic origin. Consequently, erythroid protoporphyrin production either in the marrow or in the circulating erythrocytes, appears to contribute a relatively small portion of the excess protoporphyrin

formed in EPP. However, it is likely that these relative proportions are inconstant from patient to patient, as kinships have been reported in which some members have high red cell protoporphyrin with low fecal values and vice versa (9, 10). In addition, it is possible that tissues other than the liver and the erythroid cells may overproduce protoporphyrin, but if this occurs, it must be of quantitatively minor significance, as is the case in the erythroid apparatus.

The present study adds strong evidence to the existing information that in EPP, the defect in porphyrin metabolism is identifiable in at least two different tissues, namely the liver and the erythroid apparatus. It is a misnomer, therefore, to designate this condition as EPP; instead, we propose the name of erythrohepatic protoporphyria.

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