Enzyme Defects in the Porphyrias and their Relevance to the Biochemical Abnormalities in these Disorders

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Defects in enzymes of the heme biosynthesis pathway underlie the biochemical abnormalities which occur in the porphyrias. Porphyrins and porphyrin precursors are accumulated and excreted in excessive amounts because of the enzyme defects. This is illustrated by studies in protoporphyria and variegate porphyria, disorders in which the biochemical abnormalities indicate a defect(s) in the terminal part of the heme biosynthesis pathway. The activity of heme synthease (ferrochelatase), which catalyzes the chelation of ferrous iron to protoporphyrin, is deficient in tissues of patients with protoporphyria. This causes protoporphyrin to be accumulated and excreted excessively. In variegate porphyria protoporphyrinogen oxidase, which catalyzes the oxidation of protoporphyrinogen to protoporphyrin, appears to be defective. As a result, protoporphyrinogen may be excreted in increased amounts in bile, where it is subsequently auto-oxidized to protoporphyrin. The following questions have arisen as a result of the demonstrations of enzyme defects in tissues of patients with porphyria: (1) Will different defects in the same enzyme be found among patients who fulfill the clinical and biochemical criteria for diagnosis of a specific porphyria? That is, does genetic heterogeneity exist in each of the porphyrias? (2) Why do some patients with an enzyme defect not have biochemical abnormalities? (3) Why is one type of tissue, usually the liver, the major site of expression of the biochemical abnormality, when the enzyme defect can be demonstrated in all tissues?

The porphyrias are a group of disorders in man in which inborn errors of heme biosynthesis cause excessive accumulation and excretion of porphyrins and porphyrin precursors (Fig 1). Studies over the past decade have shown that the biochemical abnormalities reflect enzyme defects in the heme biosynthesis pathway. For example, the livers of patients with acute intermittent porphyria have deficient activity of uroporphyrinogen I synthase [1], which catalyzes the conversion of porphobilinogen to uroporphyrinogen. As a result, δ-amino-levulinic acid and porphobilinogen are excreted in increased amounts in the urine. The enzyme is not defective in the livers of patients with variegate porphyria and porphyria cutanea tarda [1], which have patterns of abnormal porphyrin excretion different from that in acute intermittent porphyria. The defect in uroporphyrinogen I synthase has also been demonstrated in cultured skin fibroblasts and red cell hemolysates from patients with acute intermittent porphyria [2–6], reflecting the genetic nature of the disease.

Enzyme defects have been defined in the other porphyrias as well [7–28]. The current state of information is summarized in Table I. My laboratory has investigated protoporphyria and variegate porphyria (Table II), disorders in which the increase in fecal protoporphyrin indicates there is a defect(s) in the

terminal portion of the heme biosynthesis pathway. These studies illustrate some of the methods which have been used to investigate enzyme defects in the prophyrias and point out the questions which have been raised about their biochemical expression.

MATERIALS AND METHODS

Patient Material

Each of the patients with protoporphyria or variegate prophyria who participated in the studies had the typical clinical, biochemical, and genetic features of the disorder (Table II). All specimens were obtained from the patients after informed consent.

Liver tissue for measurement of heme synthase activity was obtained from 7 patients with protoporphyria who were undergoing evaluation for hepatobiliary disease. The features of hepatobiliary disease in protoporphyria are discussed in detail elsewhere [29,30]. Ths tissue was obtained by percutaneous needle biopsy in 6 patients, and by surgical biopsy at minilaparotomy in 1 patient. Histological evaluation of a portion of the biopsy specimen showed normal features in 2, mild portal inflammation in 2, and cirrhosis in 3. Liver tissue was also obtained from 10 control subject. Nine individuals had percutaneous needle biopsies for evaluation of abnormalities in liver function tests, for investigation of fever of unknown origin and granulomatous disease, or because they were being considered for methotrexate therapy of psoriasis. One brain-dead subject who became a kidney donor had tissue obtained immediately after death. The control specimens were either normal histologically or had minimal abnormalities. The assay of heme synthase activity was done on freshly obtained tissue in 8 cases, and on tissue which had been frozen at −70°C in 9 cases. The activity in freshly obtained tissue was not discernibly different from that in frozen tissue, either for the patients or controls.

Punch biopsies of skin (4 mm) were obtained from 8 patients with protoporphyria, 5 patients with variegate prophyria, and 8 age-matched normal individuals in order to establish lines of cultured fibroblasts. Fibroblast cultures were also initiated from skin biopsies of 3 sets of parents of children with protoporphyria.

Fibroblast Culture

Fibroblasts from the patients and normal individuals were cultured in parallel. In order to obtain a sufficient number of cells for enzyme assay, lines in the 5th to 20th passage were grown to confluence in 690-cm² roller bottles (Bellco Glass, Vineland, NY) with Eagle's minimum essential medium supplemented with 1% nonessential amino acids, 10% fetal calf serum (Flow Laboratories, Rockville, MD) and 100 μg of kanamycin per milliliter. The confluent cells were rinsed with calcium-free, magnesium-free phosphate-buffered saline (pH 7.4), harvested with 0.25% trypsin (Grand Island Biological, Grand Island, NY) into phosphate-buffered saline, centrifuged for 10 min at 1400 ×g, and washed. For measurement of heme synthase activity, the cells were resuspended in 0.25 M sucrose containing 0.05 M Tris HCl (pH 7.5) and sonicated 3 times at 100 W-sec for 20 sec. For measurement of protoporphyrinogen oxidase activity, the cells were resuspended in 0.02 M Tris HCl (pH 8.7) containing 20 mg of Tween-20 per milliliter and sonicated in the same manner.

Measurement of Heme Synthase Activity

Heme synthase activity was measured using a radiochemical assay [7,12]. When the assay was done on sonicated fibroblasts, the reaction mixture contained 1 to 3 mg of cell protein, 1 to 2 μCi of radioactive iron (New England Nuclear, Boston, MA), 25 μM ferrous sulfate, 25 μM protoporphyrin, 3.5 mM ascorbic acid, 40 mg of Tween-20, and 233 mM Tris HCl (pH 7.5) in a final volume of 4 ml. When done on liver tissue, the reaction mixture contained 3.0 to 11.7 mg wet liver weight that had been homogenized and then sonicated in 0.25 M sucrose—0.05 M Tris

HCl (pH 7.5), and the concentrations of ferrous sulfate and protoporphyrin were increased to 125 uM.

Nonenzymatic formation of heme was measured with a tissue blank prepared by boiling a portion of the tissue sonicate for 10 min. The level of nonenzymatic heme formation was subtracted from the rate of heme formed by viable tissue to obtain the level of enzymatic heme formation.

Measurement of Protoporphyrinogen Oxidase Activity

Protoporphyrinogen oxidase activity was measured using a fluorometric assay [23,31]. The reaction mixture contained 0.5 to 2.0 mg of cell protein, 1 mM EDTA, 100 mM Tris HCl (pH 8.7), 20 mg of Tween-20, and 50 uM protoporphyrinogen in a final volume of 1.5 ml. Protoporphyrinogen was generated by reduction of protoporphyrin with sodium amalgam under nitrogen in the dark. A nonenzymatic control prepared by heating a portion of the cell sonicate at 75°C for 15 min, a reagent blank, and a protoporphyrin standard were run simultaneously. The assay was started by addition of protoporphyrinogen to the untreated tissue preparation and the nonenzymatic control. Each flask

was incubated at 37°C in the dark in room air. At 20-min intervals for 1 hr of incubation, 0.1 ml of reaction mixture was removed and added to 2.9 ml of a solution that contained 5 mM glutathione, 1.0 mM EDTA, and 100 mM Tris HCl at a pH of 8.7. The fluorescence-emission intensity at 635 nm was measured with a Farrand spectrofluorometer (4818 photomultiplier tube) using an excitation wavelength of 405 nm. The concentrations of protoporphyrin in the sample solution and nonenzymatic control solution were determined by comparison to the protoporphyrin standard. The difference in protoporphyrin concentration between these solutions represented protoporphyrin that had been generated enzymatically.

RESULTS

The fact that red cell and fecal protoporphyrin levels are increased in patients with protoporphyria points to a defect in heme synthase (ferrochelatase) activity. Heme synthase activity in liver tissue obtained from patients with protoporphyria was significantly reduced compared to that in control liver tissue (Table III). The activity was reduced irrespective of the liver histology. Notably, however, the most severe reduction in activity (to 91 and 173 pmol heme/mg protein/hr) was found in tissue from the 2 patients with severe cirrhosis and massive pigment deposition.

In order to provide additional evidence that heme synthase activity is defective in protoporphyria, enzyme activity in cultured skin fibroblasts from patients was compared to that in cells from normal individuals. Cultured fibroblasts are an ideal tissue to examine since they are a homogeneous population of cells. Moreover, since they are cultured for several generations before assay, circulating factors which might influence the enzyme activity are eliminated. The results confirmed that heme synthase is defective in protoporphyria (Table IV).
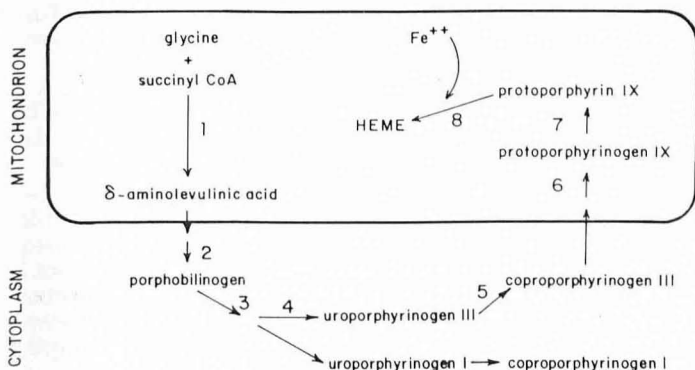
HEME BIOSYNTHESIS PATHWAY

FIG 1. Heme biosynthesis pathway. Enzymes which catalyze the steps in the pathway are as follows: (*1*) ALA synthase, (*2*) ALA dehydrase, (*3*) uroporphyrinogen I synthase, (*4*) uroporphyrinogen III cosynthase, (*5*) uroporphyrinogen decarboxylase, (*6*) coproporphyrinogen oxidase, (*7*) protoporphyrinogen oxidase, (*8*) heme synthase, (ferrochelatase).

TABLE III. *Liver heme synthase activity in patients with protoporphyria*

	No. of Patients	pmol Heme/mg protein/hr	
Protoporphyria	7	559 ± 186	
Control	10	2078 ± 298	$P < 0.01$

Mean ± SEM

TABLE I. *Enzyme defects in the porphyrias*

Disorder	Enzyme defect	Tissue where abnormality documented
Acute intermittent prophyria	Uroporphyrinogen I synthase	All tissues examined [1–6][a]
Protoporphyria	Heme synthase	All tissues examined [7–12]
Hereditary coproporphyria	Coproporphyrinogen oxidase	All tissues examined [13–15]
Porphyria cutanea tarda		
Familial	Uroporphyrinogen decarboxylase	All tissues examined [16,17]
Sporadic	Uroporphyrinogen decarboxylase	Liver only ? [18–20]
Variegate porphyria	Heme synthase ?	Bone marrow [21]; Fibroblasts [22]
	Protoporphyrinogen oxidase ?	Fibroblasts [23]
Congenital erythropoietic	Uroporphyrinogen III cosynthase/ Uroporphyrinogen I synthase imbalance	Red cells [24,25] Fibroblasts [26]
Unnamed	ALA dehydrase	Red cells [27,28]

[a] References are indicated by the numbers in brackets.

TABLE II. *Manifestations of protoporphyria and variegate porphyria*

	Protoporphyria	Variegate porphyria
Inheritance	Autosomal dominance	Autosomal dominance
Clincial features	Photosensitivity	Skin fragility
	Hepatobiliary disease	Neuropsychiatric symptoms
Biochemical abnormalities		
Erythrocytes	↑ Protoporphyrin	None
Feces	↑ Protoporphyrin	↑ Protoporphyrin, protoporphyrin-peptides, and coproporphyrin
Urine	None	↑ δ-Aminolevulinic acid, porphobilinogen, uroporphyrin, and coproporphyrin during acute attack

TABLE IV. *Heme synthase activity in cultured skin fibroblasts from members of 3 families with protoprophyria*

	No.	pmol Heme/mg protein/hr
Children with protoprophyria	5	4.0 ± 0.8[a]
Parents with enzyme defect	3	5.8 ± 2.7[a]
Normal parents	3	26.3 ± 1.9
Normal controls	8	24.9 ± 4.5

Mean ± SEM

[a] Significant difference from control ($P < 0.01$).

TABLE V. *Heme synthase and protoporphyrinogen oxidase activities in cultured skin fibroblasts from patients with variegate porphyria*

	No.	Heme synthase	Protoporphyrinogen oxidase
		pmol heme/mg protein/hr	pmol protoporphyrin/mg protein/hr
Variegate porphyria	5	36.4 ± 6.1	904 ± 132[a]
Normal control	5	32.4 ± 5.9	2120 ± 250

Mean ± SEM

[a] Significant difference from control ($P < 0.005$).

Since protoporphyria is inherited as an autosomal dominant disease, heme synthase activity should be defective in the tissues of one of the parents of a patient with protoporphyria unless the disease represents a new mutation. Cultured fibroblasts were available from 3 families in which protoporphyria was present. In each family, 2 or more children were affected with the disease. Heme synthase activity was diminished in cultured fibroblasts from one parent in each of the families, whereas that in cells from the other parent was normal (Table IV), consistent with a disease that has a dominant mode of inheritance. The enzyme defect was found in a parent from one family even though he had no biochemical or clinical manifestations of protoporphyria.

Although the biochemical abnormalities in variegate porphyria are different from those in protoporphyria (Table II), the fact that fecal protoporphyrin is constantly elevated suggests there is also a defect in the terminal portion of the heme biosynthesis pathway in this disorder. Heme synthase and protoporphyrinogen oxidase activities were assayed in cultured skin fibroblasts from patients with variegate porphyria and compared to normal levels. Heme synthase activity was normal, whereas protoporphyrinogen oxidase was reduced to 43% of normal (Table V).

DISCUSSION

These studies illustrate how enzyme defects underlie the biochemical abnormalities which occur in the porphyrias. Heme synthase activity was shown to be deficient in liver tissue and cultured skin fibroblasts from patients with protoporphyria. Other investigators have demonstrated the enzyme defect in bone marrow cells and peripheral blood cells [8,9,11]. As a result of the enzyme defect, protoporphyrin accumulates in heme-forming cells, principally the developing red cell. As the red cell enters the circulation, the protoporphyrin diffuses out of the cell and binds to plasma proteins (Fig 2). It is subsequently taken up by the liver and excreted into bile, accounting for the high levels of fecal protoporphyrin which occur in patients with protoporphyria.

The biochemical abnormalities observed in variegate porphyria (Table II) are more difficult to explain on the basis of the enzyme defect we have found. A defect in protoporphyrinogen oxidase should cause an accumulation of protoporphyrinogen, not of protoporphyrin. However, protoporphyrinogen rapidly and spontaneously oxidizes to protoporphyrin when exposed to air. Thus, protoporphyrinogen which is excreted in excess

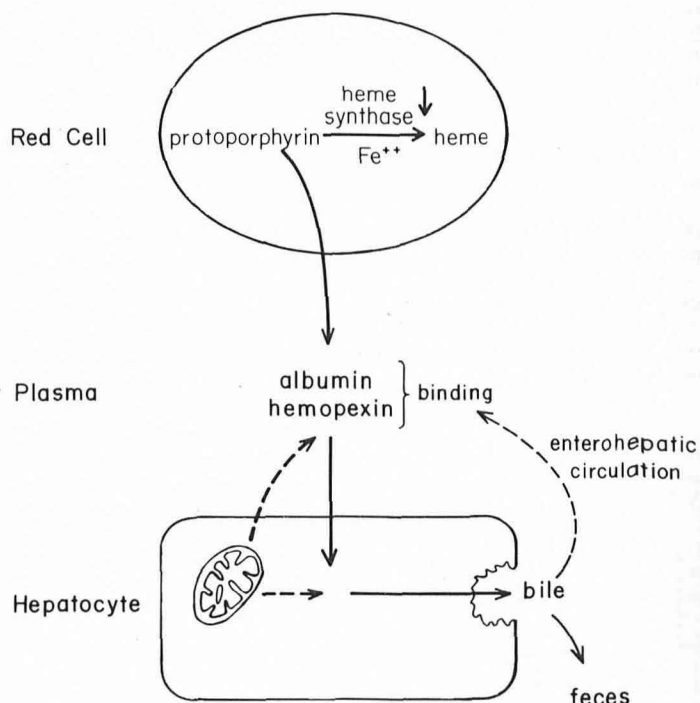# PORPHYRIN METABOLISM IN PROTOPORPHYRIA

FIG 2. Postulated basis for abnormal porphyrin metabolism in protoporphyria. As a consequence of deficient heme synthase activity, protoporphyrin is accumulated in tissues and is excreted in excessive amounts in the feces. The red cell is the primary source of the excess protoporphyrin, with a variable contribution from the hepatocyte.

amounts by the liver would oxidize to protoporphyrin in bile or feces (Fig 3), accounting for the fact that fecal protoporphyrin is increased in patients with variegate porphyria. An additional factor must be present during acute attacks, since the intermediates of the heme biosynthesis pathway which precede protoporphyrinogen are also excreted in excessive amounts. The activity of hepatic ALA synthase, which is the role-limiting enzyme in hepatic heme biosynthesis, is increased during acute attacks [1]. As a result, all the intermediates in the pathway which precede the enzyme block may be formed and excreted in increased amounts (Fig 3).

In contrast to our finding that heme synthase activity is normal in cultured fibroblasts from patients with variegate porphyria, Becker and co-workers found approximately 50% reduction in heme synthase activity in cultured fibroblasts and bone marrow cells from patients with the disorder [21,22]. They have postulated that an abnormality in heme synthase activity may be common to protoporphyria and variegate porphyria. There is no ready explanation for the discrepancy between their studies and ours. Although this may be due to differences in methodology, it is conceivable that some patients have a defect only in protoporphyrinogen oxidase, as we observed, whereas others have a defect in both protoporphyrinogen oxidase and heme synthase. Further studies in more pedigrees with the disease are required to answer this question.

The nature of the enzyme defect has not been well-characterized in any of the porphyrias. Recently, uroporphyrinogen synthase has been purified by Anderson and Desnick [32], and the enzyme abnormality in acute intermittent porphyria has been investigated. Preliminary data indicate that the enzyme deficiency in most patients results from a structural gene defect [33]. It will be of interest to learn if more than one defect in uroporphyrinogen synthase can cause acute intermittent porphyria (in other words, is there genetic heterogeneity in the

PORPHYRIN METABOLISM IN VARIEGATE PORPHYRIA

Remission

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        ALA
glycine SYNTHASE                                    PROTOPORPHYRINOGEN ↓
                                                        OXIDASE
glycine    →→→    ALA  →  PBG  →  →  →  protoporphyrinogen  →→→  protoporphyrin  →  heme
succinyl-CoA
                                              ↘
                                            bile  →  nonenzymatic oxidation
                                                      to protoporphyrin
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Relapse

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           ALA
glycine  ↑↑ SYNTHASE                                  PROTOPORPHYRINOGEN ↓
                                                         OXIDASE
glycine    →→→    ALA  →  PBG  →  →  →  protoporphyrinogen  →→→  protoporphyrin  →  heme
succinyl-CoA   └_____┘                 ↘
                         ↓                             bile  →  nonenzymatic oxidation
                       urine                                    to protoporphyrin
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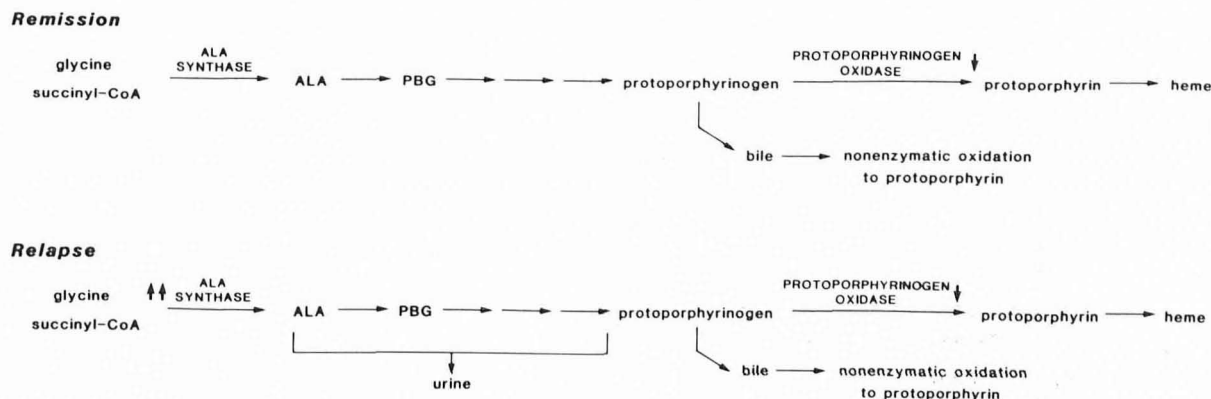
FIG 3. Postulated basis for abnormal porphyrin metabolism in variegate porphyria. As a consequence of deficient protoporphyrinogen oxidase activity, protoporphyrinogen is excessive amounts in bile. Nonenzymatic oxidation of protoporphyrinogen to protoporphyrin subsequently occurs, explaining why fecal protoporphyrin is constantly elevated in this disorder. During acute attacks, hepatic ALA synthase markedly increases, and the intermediates of the pathway which precede protoporphyrinogen are also excreted in increased amounts.

disorder). This is likely to be the case in several of the porphyrias.

Enzyme activity is defective in the tissues of some individuals who do not have biochemical manifestations of porphyria, as illustrated by other studies in parents of children affected with protoporphyria (Table IV) and by studies of other investigators [2,17]. Moreover, biochemical manifestations in individual patients vary from one period to another. Thus, other factors are important in causing biochemical expression of the disease. The requirement for hepatic heme biosynthesis is one such factor. A greater demand for heme biosynthesis may necessitate an increase in ALA synthase activity. This will increase the formation of the intermediates which precede the enzyme defect and enhance the biochemical abnormalities. ALA synthase activity is increased markedly in the livers of patients with variegate porphyria, acute intermittent porphyria, and hereditary coproporphyria during acute attacks [1,34–37]. When the patients are in remission, hepatic ALA synthase activity is lower, and the excretion of porphyrins and porphyrin precursors is less [1,37].

Other factors may modify the enzyme defects. For example, the level of iron influences the degree to which protoporphyrin accumulates in fibroblasts cultured from patients with protoporphyria [38]. Ferrous iron also appears to inhibit uroporphyrinogen decarboxylase activity in crude liver extracts [39]. Since patients with porphyria cutanea tarda characteristically have hepatic hemosiderosis, this may bring out the disease in patients who already have reduced enzyme activity. Further identification of factors which modify the enzyme defects will increase our understanding of the fluctuations which occur in the biochemical manifestations of the prophyrias.

The defect in heme synthase activity has been found in all tissues from patients with protoporphyria. Similarly, the enzyme defects in acute intermittent porphyria, hereditary coproporphyria, and familial porphyria cutanea tarda have been found in all tissues examined. This reflects the genetic nature of these disorders. However, it raises the question why the red cell appears to be the principal site of expression of the biochemical abnormality in protoporphyria, whereas the liver is the major site in most of the other porphyrias [40]. One possibility is that enzyme defects in nonhepatic tissues do not reduce the activities of most of the enzymes to critical levels such that porphyrins and porphyrin precursors accumulate. In cultured skin fibroblasts, for example, protoporphyrinogen oxidase activity is high compared to that of ALA synthase, uroporphyrinogen I synthase, and heme synthase (Fig 4). Thus, a defect in

HEME PATHWAY ENZYMES IN CULTURED FIBROBLASTS

pmol porphyrin equivalents/mg protein/hr at 37°c

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glycine     3.0, 4.5, 5.4          ?          3.8, 11.9
succinyl-CoA  →→→  ALA  →→→  porphobilinogen  →→→  →→→  uroporphyrinogen
            ALA          ALA          URO I    URO III
          SYNTHASE    DEHYDRASE     SYNTHASE  COSYNTHASE

   2088                      545                     2120
  →→→  coporphyrinogen  →→→  protoporphyrinogen  →→→  protoporphyrin
  URO                  COPRO                    PROTO
DECARBOXYLASE         OXIDASE                   OXIDASE

        32.4
       →→→  heme
       HEME
     SYNTHASE
```
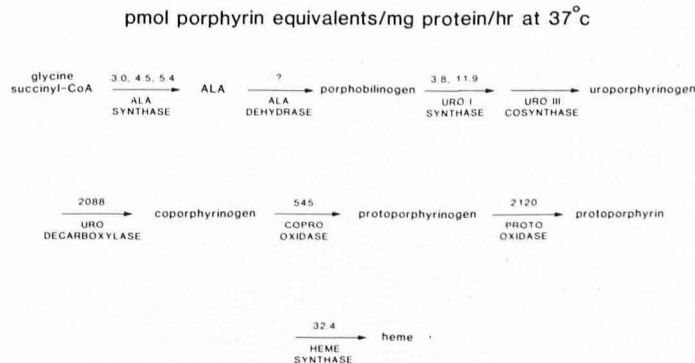
FIG 4. Specific activities of heme pathway enzymes in cultured skin fibroblasts. Relative activities of the enzymes are shown in terms of porphyrin equivalents generated. When more than one value is given, the data were generated in different laboratories (see references in Table I).

protoporphyrinogen oxidase does not cause porphyrin metabolism to be altered in these cells, whereas a defect in heme synthase will [23,38].

The past decade has brought progress in our understanding of the pathogenesis of the biochemical abnormalities in the porphyrias. Several interesting and important questions have arisen as a result of this information and require further investigation.

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